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pyrimidine A Spinks

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2-p-AMINOBENZENESULPHONAMIDO-4:6-DIMETHOXYPYRIMIDINE EXPERIMENTAL EVALUATION

BY

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(Received January 16 1947)

The introduction of an a-pyridyl residue into the molecule of sulphanilamide by Ewins and Phillips (1937) was a major advance in the development of sulphanilamide therapy, since not only was there produced a marked improvement in intrinsic antibacterial activity, but the new substance, sulphapyridine, was the starting point for an extensive research on the preparation of sulphanilamide drugs derived from other heterocyclic systems. The pyrimidine ring system has been particularly fruitful and at least three drugs derived from this nucleus are in common use sulphadiazine, sulphamerazine, and sulphameza-They are characterized by antibacterial activity of a high order against a wide range of organisms, by rapid and efficient absorption from the gastrointestinal tract, and by a degree of persistence in the blood which provides economy in use The work of Bell and Roblin (1942) suggests that the discovery of sulphanilamide drugs possessing a higher intrinsic antibacterial activity than that exhibited by the sulphapyrimidine group is becoming more remote improvement in sulphanilamide therapy must therefore arise from research devoted to the production of drugs exhibiting reduced toxicity and enhanced persistence in the blood. We have interested ourselves for some time particularly in the latter aspect, and have found this property to be outstanding in sulphanilamides derived from 2-amino-4 6-dialkoxypyrimidines A series of such compounds has been prepared (Rose and Tuey, 1946) and we propose to publish in due course detailed pharmacological and bacteriological findings This memoir is concerned with the parent on these substances as a class compound 2-p-aminobenzenesulphonamido-4 6-dimethoxypyrimidine (sulphadimethoxypyrimidine), which is the most effective of the many homologues which have been examined

$$NH_2 \longrightarrow SO_2 NH < N \longrightarrow OMe$$

While this paper was in preparation our attention was drawn to the researches of van Dyke, Tupikova, Chow, and Walker (1945), who, in the course of an

extensive study of sulphapyrimidines, have examined some of the 4 6-dialkoxy derivatives with which we have been concerned. In the main, these authors confirm our findings

We describe the pharmacology and *in vitro* antibacterial properties of sulphadimethoxypyrimidine, the combination of these factors in therapeutic activity, the toxicity and the physico-chemical properties A note on the unusual properties of the acetyl derivative of sulphadimethoxypyrimidine is included.

The clinical activity and pharmacology of the drug are now being investigated at Crumpsall Hospital, Manchester, and will be reported later by the workers concerned

PHARMACOLOGY

The blood concentrations attained by sulphadimethoxypyrimidine have been examined in the mouse, rat, rabbit, dog, chick, sheep and calf For data on the last two species we are indebted to Mr J Francis, of our Veterinary Research Laboratories

In the mouse—Absorption was examined by the standard technique already described (Rose and Spinks, 1946) Three mice received 250 mg/kg, by stomach tube as a 1 g/100 ml solution of the sodium salt, and the drug was estimated at intervals in pooled tail blood by the method of Rose and Bevan (1944) In a series of 14 analyses of 0.05 ml samples of blood containing 2 to 20 mg/100 ml, the mean recovery was 98.5 per cent \pm standard deviation 6.95 per cent, limit of error for a probability level of 0.05, \pm 4.00 per cent.

The single experiment was repeated 22 times, so that 66 animals contributed to the mean results (Table I), in Fig 1 the mean absorption curve is compared with that of sulphamerazine

TABLE I
BLOOD CONCENTRATIONS OF SULPHADIMETHOXYPYRIMIDINE
250 mg /kg in groups of 3 mice

	Mean blood concentrations in mg /100 ml after										
20 min	40 min	1 hr	1 5 hr	2 5 hr	3 5 hr	5 hr	7 hr	24 hr			
11 6	12 7	13.2	14 1	13 8	14 4	14.3	14 0	94			
17 7	21 5	21 1	20.2	20 4	19 3	19 1	170	94			
14 1	19 1	18 8	199	170	20 6	20 3	186	77			
	11 6 17 7	20 min 40 min 11 6 12 7 17 7 21 5	20 min 40 min 1 hr 11 6 12 7 13.2 17 7 21 5 21 1	20 min 40 min 1 hr 1 5 hr 11 6 12 7 13.2 14 1 17 7 21 5 21 1 20.2	20 min 40 min 1 hr 1 5 hr 2 5 hr 11 6 12 7 13.2 14 1 13 8 17 7 21 5 21 1 20.2 20 4	20 min 40 min 1 hr 1 5 hr 2 5 hr 3 5 hr 11 6 12 7 13.2 14 1 13 8 14 4 17 7 21 5 21 1 20.2 20 4 19 3	20 min 40 min 1 hr 1 5 hr 2 5 hr 3 5 hr 5 hr 11 6 12 7 13.2 14 1 13 8 14 4 14.3 17 7 21 5 21 1 20.2 20 4 19 3 19 1	20 min 40 min 1 hr 1 5 hr 2 5 hr 3 5 hr 5 hr 7 hr 11 6 12 7 13.2 14 1 13 8 14 4 14.3 14 0 17 7 21 5 21 1 20.2 20 4 19 3 19 1 17 0			

In an earlier paper (Rose and Spinks, 1946) the expression C7, which is the time required for the blood concentration to fall from that attained 7 hours after dosing to two-thirds of this figure, was suggested as a convenient means of defining the rate of disappearance of a sulphonamide from the blood Sulphadimethoxypyrimidine is clearly more persistent than sulphamerazine, the values

of C7 taken from the mean curves being 162 and 73 hours respectively By obtaining the characteristic values from individual curves (Rose and Spinks, 1946) and submitting the results to statistical analysis, the difference is readily shown to be decisive (P < 0.01) Sulphadimethoxypyrimidine is thus 22 times as persistent as sulphamerazine in mice, the term persistence being taken to mean the retention of free drug in the blood. The new drug reaches fairly high concentrations, the mean of the maximum concentrations from individual curves is 15.3 ± 1.25 mg/100 ml, which is higher than the corresponding figure for

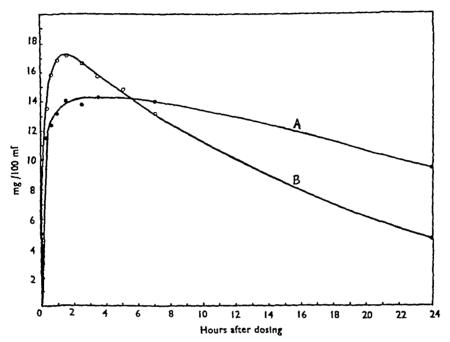


Fig 1—Blood concentrations in mice of sulphadimethoxypyrimidine (A) and sulphamerazine (B), following the administration of 250 mg/kg orally

sulphanilamide, sulphapyridine, or sulphathiazole, although lower than that for sulphamezathine, sulphamerazine, or sulphadiazine. Sulphadimethoxypyrimidine is rather slowly absorbed, the time at which the maximum blood concentration is attained, taken from the mean curve, being 220 minutes. Using the method of statistical analysis already described (Rose and Spinks, 1946), the drug can be shown to be significantly more slowly absorbed than any of the other three sulphapyrimidines.

The blood concentrations attained by the drug following intraperitoneal and subcutaneous administration of 250 mg/kg to mice are shown in Table I. From these it must be concluded that the drug is more rapidly and more completely absorbed by these routes than when given orally. Persistence in the blood is again marked, indeed there is no significant difference in this respect between the intraperitoneal and oral routes. The mean maximum blood concentration

of individual intraperitoneal curves (20.7 mg / 100 ml) is decisively higher than that of individual oral curves (15.3 mg / 100 ml), and the difference between the mean times of attaining these maximum values is also decisive. Owing to the difficulty of drawing some of the individual subcutaneous curves, no statistical comparison has been attempted in this case.

Sulphadimethoxypyrimidine can be detected in the blood of mice for several days after the administration of a single oral dose of 250 mg/kg. Blood concentrations recorded in a typical experiment with three mice were

Time (hr)	2	22	27	42	46	66	71	73	89	139
mg /100 ml	13 2	13 85	13 4	5 0	531	40	4 2	1 2	0 25	0 14

The maintenance of high concentrations for so extended a time suggested that repeated dosing might have a cumulative effect, giving dangerously high concentrations. This possibility was examined by administering two oral doses of 250 mg/kg at an interval of

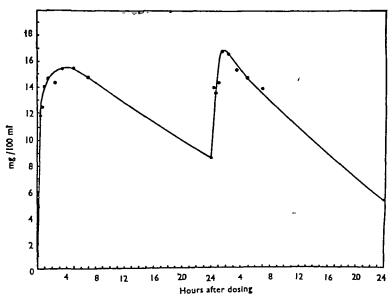


Fig 2—Blood concentrations of sulphadimethoxypyrimidine following the administration to mice of two oral doses of 250 mg/kg at an interval of 24 hours

24 hours to a group of three mice and estimating the concentration of free drug in the blood at suitable intervals after each dose. The experiment was repeated ten times, with the results shown in Fig. 2. Clearly there is no marked rise in maximum blood level on such repeated dosing. This has been confirmed by statistical treatment, which shows that there is no significant difference between the mean maximum concentrations, but there are decisive differences between the mean times at which these maxima were attained and between the respective rates of disappearance of the drug from the blood stream. We have no explanation to offer for the greater rapidity with which the second dose is absorbed but it may be that this phenomenon is associated in some way with the lower persistence

Tissue concentrations in mice have been estimated following the usual dose of 250 mg/kg, three mice being used for each point recorded in Table II In Table III the corresponding values for sulphamezathine are given for comparison, these have not been previously recorded. With both drugs, the concentrations in tissues approximated to those in blood in magnitude and persistence. Figures showing tissue distribution in the nephrectomized cat are given in the following section.

TABLE II
TISSUE CONCENTRATIONS OF SULPHADIMETHOXYPYRIMIDINE
250 mg /kg orally in groups of 3 mice

í

Tissue	Concentrations in mg /100 g of wet tissue after									
	30 min	2 hrs	4 hrs	6½ hrs	19 hrs	31 hrs	48 hrs	72 hrs	96 hrs	
Lung Liver Kidney Spleen Fat	14 5 15 1 14 1 7 0	16 9 13 6 12 9 6 8 9 8	19 6 12 9 12 2 9 5	17 6 15 0 7 9 9 5	16 3 14 6 8 9 10 8	10 1 4 0 6 7 5 5	2 6 2 7 2 7 1 6	3 8 2 5 2 6 0 5	2 1 1 1 0 5 0 6	

The urinary excretion of the drug in the mouse has been examined in two groups of three animals. Following the administration of 250 mg/kg orally, 46 and 32 per cent of the administered drug was excreted over three days in the two groups, and of these amounts 39 and 44 per cent respectively were acetylated. These results confirm the conclusion drawn from estimations of blood concentration after intraperitoneal injection, namely, that the drug is poorly absorbed in the mouse. The observation that, although much more persistent than sulphamezathine or sulphamerazine, sulphadimethoxypyrimidine gives lower blood concentrations than either of these compounds, can be explained on the same basis

Experiments to determine the extent of conjugation of the new drug in the blood stream of the mouse are described below in the section dealing with acetylsulphadimethoxy-pyrimidine

TABLE III
TISSUE CONCENTRATIONS OF SULPHAMEZATHINE
250 mg/kg orally in groups of 3 mice

Tissue		Concentrations in mg/100 g of wet tissue after								
	30 min	2 hrs	4 hrs	7 hrs	24 hrs					
Lung Liver Kidney Spleen Fat	21 8 18 0 19 9 14 8 10 7	17 1 18 8 20 8 11 1 6 6	12 3 10 7 17 3 6 3 7 1	65 31 132 42 53	23 30 16 14 19					

In species other than the mouse—The blood concentration-time curves presented have in each case been chosen as typical from at least three available in the particular species. The drug was given orally as a solution of the sodium salt in the following amounts dog, calf, sheep, 100 mg/kg, rabbit, 150 mg/kg, rat chick 200 mg/kg. The

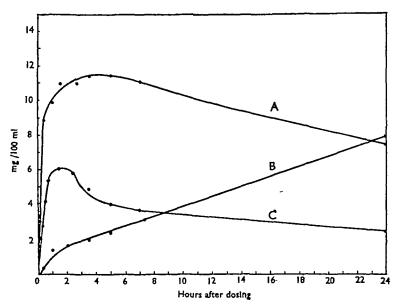


FIG 3—Blood concentrations of sulphadimethoxypyrimidine following the oral administration of 200 mg/kg to the rat (A) and chick (B) and of 150 mg/kg to the rabbit (C)

resultant blood concentrations are given in Figs 3 and 4. In the rabbit, the urinary excretion of the drug has been measured, and the amounts appearing as free amine and in conjugated form estimated (Table IV). Rabbit A, which received 150 mg/kg of the drug, excreted approximately 80 per cent in the urine, of which some 47 per cent was conjugated

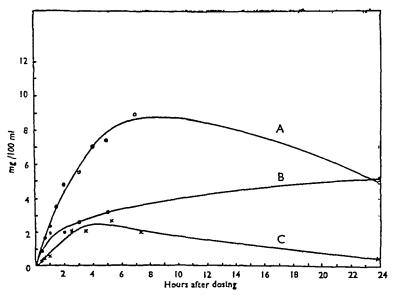


Fig 4—Blood concentrations of sulphadimethoxypyrimidine following the oral administration of 100 mg./kg to sheep (A), calf (B) and dog (C)

TABLE IV

EXCRETION OF FREE AND CONJUGATED SULPHADIMETHOXYPYRIMIDINE IN THE URINE OF TWO RABBITS

R	Rabbit A (1 2 kg , dose 180 mg)					bit B (2 1	7 kg , do	ose 217 mį	g)
Free drug Total drug				drug		Free drug		Total drug	
Day	mg	% of dose	mg	% of dose	Day	mg	% of dose	mg	% of dose
1 2 3 4 + 5	62 7 5 7 5 8 3 1	34 8 3 2 3 2 1 7	120 3 13 8 6 4 5 9	66 8 7 7 3 6 3 3	1 2 3 4 + 5	35 5 5 4 7 6 2 2	16 3 2 5 3 5 1 0	134 9 14 8 13 7 4 3	62 1 6 8 6 3 2 0
Total	77 3	42 9	146 4	81 4	Total	50 7	23 3	167 7	77 2

The proportion of total drug excreted by rabbit B, which was given a smaller dose of 100 mg/kg, was similar, but the percentage conjugated was much higher (70 per cent) Despite the high recovery, indicative of efficient absorption from the gastro-intestinal tract, the maximum blood concentrations attained in the rabbit were appreciably lower than those reached in the rat and mouse although a similarly high degree of persistence was observed. The expression C7, obtained from Fig. 3, had a value of 14 hours for the rabbit, as against 17 hours for the rat and 16.5 hours for the mouse. In the sheep, calf, and chick the drug was slowly absorbed but persisted well, particularly in the last two. Absorption was poor in the dog and removal from the blood stream rapid (C7=2 hours). Conjugation in rabbit and sheep is described in the section dealing with acetylsulphadimethoxypyrimidine.

The tissue distribution of the drug in nephrectomized cats has been estimated using the technique of Fisher, Troast, Waterhouse, and Shannon (1943) The results recorded in Table V are the means of three experiments and show the tissue/plasma ratio, they are compared with the figures obtained by Fisher et al for sulphamerazine and sulphadiazine

TABLE V
DISTRIBUTION OF SULPHADIMETHOXYPYRIMIDINE IN THE BILATERALLY NEPHRECTOMIZED CAT

Drug		Vol of distri- bution (as % of						
Drug	CSF	Brain	RBC	Lung	Liver	Pancreas	Muscle	
Sulphadimethoxy- pyrimidine Sulphamerazine* Sulphamezathine*	0 23 0 38 0 31	0 23 0 35 0 21	0 12 0 45 0 53	0 40 0 56 0 60	0 68 0 76 0 63	0 35 0 47 0 44	0 24 0 39 0 45	39 45 8 82 5

^{*}Data of Fisher et al (1943)

It will be seen that sulphadimethoxypyrimidine diffuses rather less into most tissues than the other sulphapyrimidines, and markedly less into the red blood cells, this may possibly be connected with the high protein binding of the drug (vide infra)

Antibacterial action in vitro

Comparisons of the antibacterial activities of sulphadimethoxypyrimidine, sulphamezathine and sulphadiazine were made by the method of Harper and Cawston

(1945) The medium used was Wright's broth containing 10 per cent (v/v) of lysed horse blood Serial two-fold dilutions of the various sulphonamides in this medium were placed in 3 in $\times \frac{1}{2}$ in tubes in 0.5 ml amounts, and 0.5 ml of a 0.2×10^{-6} dilution in plain broth of a 24-hour culture of *Streptococcus pyogenes* Kruger strain, was added to each tube (This inoculum gave a count of approximately 5×10^{4} colonies per ml on blood-agar) The final

TABLE VI

COMPARATIVE ANTIBACTERIAL ACTIVITIES RESULTS OF 48-HOUR PLATING ON BLOOD-AGAR

Organism Streptococcus pyogenes

Sulphonomido		Control			
Sulphonamide	40,000	80,000	160,000	320,000	Control
Sulphadimethoxypyrimidine Sulphamezathine Sulphadiazine		_ ± _	+ + ±	+ + + +	+

medium thus contained 5 per cent of lysed horse blood and the concentration of the sulphonamides ranged from 1 40,000 to 1 320,000 All tubes were incubated at 37° C for 48 hours. One loopful from each tube was removed and plated upon blood-agar. The end-points were quite sharp and were recorded as — (no growth), \pm (partial growth), and + (growth equal to control). The results are shown in Table VI. It will be seen that sulphadimethoxypyrimidine was intermediate in activity between the other two compounds

Acute toxicity

When a suspension of sulphadimethoxypyrimidine was given orally to a group of 6 mice at the rate of 10~g/kg none died. The intravenous injection of solutions of the sodium salts of sulphamezathine and of sulphadimethoxypyrimidine gave the results shown in Table VII, which is a summary of two experiments. All doses were contained in a volume of 0.2~ml, and each injection took 1~min to complete

TABLE VII

THE TOXICITY OF SULPHAMEZATHINE AND SULPHADIMETHOXYPYRIMIDINE BY INTRAVENOUS INJECTION OF THEIR SODIUM SALTS

Sixteen mice in each group All mice observed for 5 days

Dose	Sulphamezathine	Sulphadimethoxy- pyrimidine
10g/kg. 09 ,, ,, 08 ,, ,, 07 ,, ,, 06 ,, ,,	16 died 16 ,, 13 ,, 2 ,, 2 ,, 0 ,,	16 died 13 ,, 12 ,, 10 ,, 4 ,, 0 ,,

Chronic toxicity

Two separate experiments were carried out in which sulphadimethoxypyrimidine was administered as an aqueous dispersion once a day to rats for 28 days, at the rate of 1 g per kg body weight per day. The total number of rats in the two experiments was 54

At the end of the period of administration of the drug the average final body weight of the treated animals was 130 per cent of their initial weight (from 110 g to 143 g), whilst the corresponding figure for equal-sized groups of matched control rats was 150 per cent

Thirteen of the treated animals died during the period of treatment, or were killed because they were losing weight and were obviously ill, usually about 14 days from the start of the experiment. In several of these rats death was undoubtedly hastened by septic broncho-pneumonia brought on by maladministration of the doses of drug, but there were other deaths among rats whose lungs appeared normal. In the majority of the latter death could be ascribed to severe central necrosis of the liver lobules, sometimes affecting the whole lobule. It should be noted that the livers of 36 of the rats showed no abnormalities. Anaemia was very marked in many of the survivors, 5 of them giving readings of 29-46 per cent haemoglobin by the Sahli method. The spleens of many of the animals showed evidence of increased destruction of red blood corpuscles.

Although damage to the kidneys had been expected on account of the low solubility of this compound, such damage was seen in only 7 of the 54 animals examined. In only one of these was it severe, consisting of massive destruction of many convoluted tubules, amounting often to complete disintegration of the cell, with loss of nuclear staining and desquamation of the cells into the lumen of the tubule

Therapeutic activity

Therapeutic experiments were carried out in mice infected with either Streptococcus pyogenes, Kruger strain (Group A), Streptococcus pneumoniae Type I, or Staphylococcus aureus The first two organisms were given intraperitoneally and the third intravenously All drugs were administered by mouth as aqueous solutions or dispersions, the desired dose

TABLE VIII

THERAPEUTIC RESULTS IN GROUPS OF 12 MICE INFECTED WITH Streptococcus pyogenes,

Streptococcus pneumoniae or Staphylococcus aureus

Oral doses of 100 mg/kg twice daily for 3 days

	Strepto pyog		Strepto pneun		Staphylococcus aureus		
Drug	No of deaths in 7 days	Mean sur- vival time in days (max 7)	No of deaths in 7 days	Mean sur- vival time in days (max 7)	No of deaths in 14 days	Mean sur- vival time in days (max 14)	
None (controls) Sulphamilamide Sulphapyridine Sulphathiazole Sulphamezathine Sulphadiazine Sulphamerazine Sulphadimethoxy- pyrimidine	12 12 11 12 12 12 11 12	0 8 1 0 1 7 1 0 2 3 3 8 4 0	12 12 12 12 10 12 —	0 8 1 2 2 8 2 1 3 6 4 3 —	12 10 9 8 5 4 —	1 6 5 0 6 1 7 2 9 4 11 2 —	

Streptococcus pyogenes Infecting dose 0 2 ml of a 1 10³ dilution of an 18-hour culture (freshly isolated from a mouse) in 5 per cent (v/v) blood-broth, given intraperitoneally Streptococcus pneumoniae Infecting dose 0 2 ml of a 1 10³ dilution of an 18-hour culture (freshly isolated from a mouse) in 5 per cent (v/v) blood-broth, given intraperitoneally

Staphylococcus aureus Infecting dose 0.2 ml of a 1 2 dilution of a plain broth culture 18 hours old, given intravenously

being contained in a volume of 0.5 ml. The drugs were administered shortly before the infection and further doses were given 7, 24, 31, 48, 55, 72, and 79 hours after the infection. The dose chosen for all the drugs in the first experiment (100 mg/kg) was selected because experience had shown that, with streptococcal and pneumococcal infections, it permitted the various compounds to be ranged in order of activity on the basis of mean survival times. Table VIII summarizes the results of these experiments

Taking all the experiments together, it will be seen that the increasing order of effectiveness of these compounds is approximately that in which they are arranged in the table

In order to simulate more closely the conditions under which these drugs are used in clinical practice, another experiment was carried out in which infection of the mice preceded drug treatment by seven hours, in this experiment the mice were infected intraperitoneally with streptococci. The results are shown in Table IX

The results of this experiment range the drugs in substantially the same order as before. In both experiments sulphadimethoxypyrimidine compares very favourably with any of the other drugs.

TABLE_IX

THERAPEUTIC RESULTS IN GROUPS OF 12 MICE INFECTED WITH Streptococcus pyogenes

Infected at 10 a m and treated at 5 p m of the same day with 500 mg drug/kg No further doses given

Drug	No of deaths in group	Mean survival time in days (maximum 7)
None (controls) Sulphanilamide Sulphapyridine Sulphathiazole Sulphamezathine Sulphadiazine Sulphamerazine Sulphamerazine Sulphadimethoxypyrimidine	12 12 12 12 12 12 12 12 12	0 8 0 8 1 5 1 0 1 5 2 3 2 5 2 8

The infecting dose was 0.2 ml of a 1 10s dilution of a culture of Streptococcus pyogenes prepared as in Table VIII

Acetylsulphadimethoxypyrimidine

Estimation and occurrence—As indicated above, sulphadimethoxypyrimidine appeared in a conjugated form in the urine of experimental animals. Since hydrolysis gave a diazotize able amine, the conjugated product was assumed to be the acetyl derivative and all estimations were made against this compound as standard. The method of Rose and Bevan (1944) proved satisfactory for the estimation of acetylsulphadimethoxypyrimidine in urine, but it yielded low recoveries when known amounts of the compound were added to blood and tissue homogenisates. Variable recoveries of the order of 60 per cent were obtained from human blood and 30 per cent or less from mouse tissues. The recovery from blood was increased to 70 per cent by hydrolysis of the conjugated drug in whole blood before the precipitation of proteins. This could not be regarded as satisfactory, and further work

showed that adequate recoveries were obtained from blood and tissue by diluting to 1 150 before precipitating protein. A suitable aliquot was then taken, hydrolysed with dilute hydrochloric acid, diazotized and coupled with $N-\beta$ -sulphatoethyl-m-toluidine. A coupling time of 30 min was required, this is greater than that of the parent compound and probably indicates breakdown of the pyrimidine ring (cf. Frisk, 1943)

Estimations of free and total drug in the blood of mice receiving 250 mg of sulphadimethoxypyrimidine/kg orally, indicated the presence of traces only of conjugated amine. In the rabbit, on the other hand, high concentrations of conjugated drug were reached (Fig 5) Similar results were obtained in the sheep, it being clear in both species that the acetyl derivative is similar in persistence to the free drug

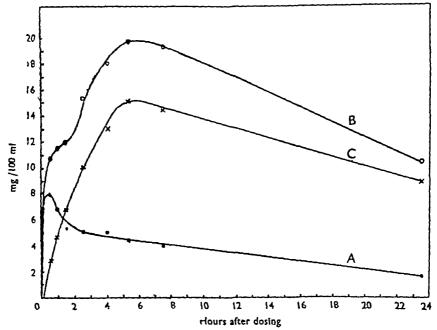


Fig 5—Blood concentrations of free (A), total (B), and conjugated (C, by difference) sulphadimethoxypyrimidine in the rabbit following the oral administration of 250 mg/kg orally

The administration of acetylsulphadimethoxypyrimidine was examined in the mouse Four groups of three mice received 250 mg/kg orally as a 1 per cent solution of the sodium salt. The mean blood concentration-time curves of free and total drug are shown in Fig 6 That the observed hydrolysis of the acetyl derivative proceeds systemically and not in the lumen of the gut was readily shown by administering it intraperitoneally, when curves very similar to those of Fig 6 were obtained. This facile hydrolysis of acetylsulphadimethoxy-pyrimidine undoubtedly accounts for its high activity, observed in the therapeutic experiments described below. In experiments with acetylsulphanilamide we found only traces of free drug in the blood following its administration in doses of 250 mg/kg, a result in agreement with its inactivity in therapeutic tests

Therapeutic activity—Only activity against Streptococcus pyogenes in mice has been examined in this instance, the infecting inoculum being similar to that used in the experiments recorded in Table VIII—The dosage regime was slightly altered in that amounts of 250 mg/kg mouse (as against 100 mg/kg mouse of the free amine) were given twice daily for three days, beginning shortly before infection—Acetyl derivatives of other sulphonamide drugs were included for comparison—Two series of experiments were made—in one

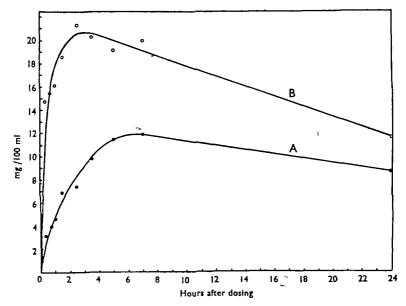


Fig 6—Blood concentrations of free (A) and total (B) sulphadimethoxypyrimidine following the oral administration in mice of 250 mg of the acetyl derivative/kg

the drugs were administered by mouth, and in the other intraperitoneally. The average survival times are indicated in Table X. Groups of 6 mice only were employed in each experiment.

Since the order of relative effectiveness of the acetyl derivatives is the same by both routes of administration, it appears that in all cases they are largely absorbed as such, and

TABLE X

THERAPEUTIC RESULTS OBTAINED IN GROUPS OF 6 MICE INFECTED WITH Streptococcus pyogenes
250 mg/kg twice daily for 3 days

D =	0	ral	Intraperatoneal			
Drug	No of deaths in 7 days	Mean survival time (max 7)	No of deaths in 7 days	Mean survival time (max 7)		
None (controls) Acetylsulphanilamide Acetylsulphanilamide Acetylsulphadiazine Acetylsulphadiazine Acetylsulphathiazole Acetylsulphamezathine Acetylsulphamerazine Acetylsulphadimethoxy- pyrimidine	6 6 6 4 6 6	08 09 09 11 32 15 08	6 6 6 6 6 6 -	08 08 08 10 23 10 —		

The infecting dose was 0.2 ml of a 1 104 dilution of a broth culture of Streptococcus pyogenes prepared as in Table VIII

absorption is not preceded by extensive hydrolysis to the free amines in the gastro-intestinal tract. This accords with the view expressed above in the case of acetylsulphadimethoxy-pyrimidine following direct estimation of blood concentrations of the free amine.

Physico-chemical properties

Solubility—The solubility determinations were made in water at 37° C by the method of Rose, Martin, and Bevan (1943), increasing pH by the addition of sodium hydroxide

The concentration of solution drug in a sample withdrawn through a filter plug into a pipette was determined colori-With the acetyl metrically derivative, de-acetylation was necessary before the colorimetric estimation, which denended upon a diazo reac-The solution, could be made bility curves so obtained are In the pH shown in Fig 7 range 60-70, the solubility of the free amine closely resembles that of sulphadiazine (Rose, Martin, and Bevan. thereafter the curve 1943) rises more steeply Acetylsulphadimethoxypyrimidine differs from acetylsulphadiazine, however, in that it is less soluble than the parent amine in the pH range 60-73, but above the latter limit the acetyl derivative exhibits the greater solubility

The acid dissociation constant — The acid dissociation constant has been measured by potentiometric titration of the saturated aqueous solution and

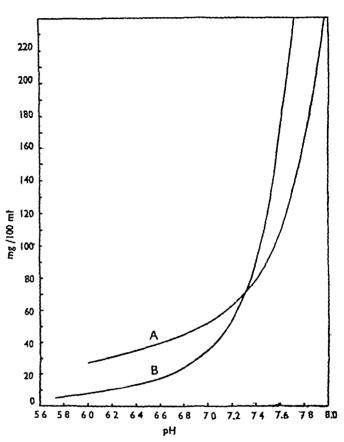


Fig 7—Solubility in water of sulphadimethoxypyrimidine (A) and of the acetyl derivative (B)

TABLE XI
PROTEIN DINDING OF SULPHADIMETHOXYPYRIMIDINE

21	Percentage of drug bound to protein						
Plasma or serum	Ultrafiltration	Dialysis					
Rat plasma Rat serum Human plasma Mouse plasma Cat plasma Sheep serum Rabbit serum Horse serum	85 87 ——————————————————————————————————	82 80 80 78 — —					

the pK_n value found to be 700 A solvent partition method gave the value 702 The pK_n values for other sulphanilamide derivatives of pyrimidine are known—e.g., sulphadiazine 648, sulphamerazine 706, sulphamezathine 737 (Bell and Roblin, 1942)

Protein binding—The association of the drug with blood proteins has been estimated by the equilibrium dialysis method of Davis (1943) and by ultrafiltration through collodion Table XI shows the results obtained with various species

It appears that sulphadimethoxypyrimidine is among the more highly bound sulphonamides, though a precise comparison is not possible, owing to the wide variation in values quoted for other drugs in the literature. The results are of the same order as the figure of 78 per cent quoted by van Dyke et al (1945) for the same compound

SUMMARY AND CONCLUSIONS

- 1 The properties of 2-p-aminobenzenesulphonamido-4 6-dimethoxypyrimidine (sulphadimethoxypyrimidine) are described, and include water solubility data for the free amine and the acetyl derivative over a range of pH
- 2 The drug is relatively non-toxic in mice and rats, rather more slowly absorbed than sulphadiazine, sulphamerazine, or sulphamezathine when given orally to mice, but markedly more persistent in the blood-stream than these three drugs Absorption data are given for other experimental animals
- 3 A high percentage of the absorbed drug is excreted by the mouse in conjugated form, but feeding the acetyl derivative (or injecting intraperitoneally) gives rise, after a few hours, to a concentration of the free amine in the blood almost equal to that attained by initial administration in the latter form
- 4 The antibacterial activity in vitro of the drug against Streptococcus pyogenes is intermediate between that of sulphamezathine and sulphadiazine, but therapeutic activity against this organism in the mouse is, in general, at least equal to or slightly better than that shown by any of the other sulphapyrimidine derivatives, in conformity with the high persistence of the drug
- 5 Acetylsulphadimethoxypyrimidine given orally or intraperitoneally to infected mice exerts an appreciable therapeutic effect, greater than that shown by the acetyl derivatives of the several other heterocyclic sulphonamides examined

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2-p-AMINOBENZENESULPHONAMIDO-4 · 6-DIMETHOXYPYRIMIDINE ABSORPTION AND EXCRETION IN MAN

BY

H G L BEVAN

WITH NOTES ON A CLINICAL TRIAL IN PNEUMONIA

BY

R W LUXTON

From the Crumpsall Hospital, Manchester
- (Received January 1 1947)

The work of Gage, Martin, Rose, Spinks, and Tuey (1947) showed that a new sulphonamide, 2-p-aminobenzenesulphonamido-4 6-dimethoxypyrimidine or sulphadimethoxypyrimidine, had an unusual persistence when administered orally to animals. This fact, in conjunction with the observation that the anti-bacterial action in vitro and in vivo was of the same order as that of sulphadiazine, suggested that the compound merited a clinical trial, which was carried out in the medical wards of Crumpsall Hospital, Manchester. It was hoped that a sulphonamide which persisted in the blood for longer periods than other sulphonamides might be effective in the treatment of pneumonia in a single dose, or at most in doses once or twice daily

METHODS

Sulphadimethoxypyrimidine was first administered to essentially normal patients, mostly convalescing from surgical and skin conditions, in doses ranging from 0.5 to 5 g. These patients were, at the time of testing, in fairly good general health and had no febrile condition, except in one patient (receiving 0.5 g.) renal function was normal. Blood concentrations and recoveries in the urine are illustrated in the Table and Fig. 1. Two patients only were used for each dose level. The drug was also administered to a series of 80 patients, mostly suffering from pneumonia. One or two of these patients had other conditions normally requiring sulphonamide therapy. The results for 30 of these patients are shown in Figs. 2, 3, and 4, those obtained from the rest of the patients were similar although several patients had not received the full course before they were transferred to other treatments, penicillin or sulphamezathine, on clinical grounds

Blood and urine sulphonamide estimations were made by the method of Rose and Bevan (1944) Twenty-four-hour collections of urine were made without preservative For the blood urea, protein, phosphatase, and bilirubin estimations the methods of King (1946), slightly modified, were used

RESULTS

Normal subjects showed a steady increase in maximum blood concentration and persistence with increasing dosage (Table, Fig 1) The maximum was

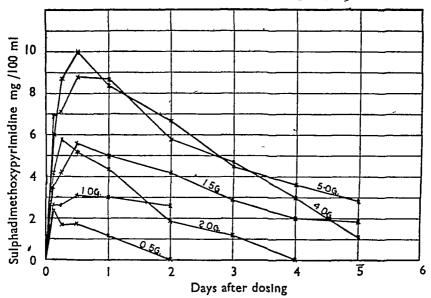


Fig 1—Blood concentrations of sulphadimethoxypyrimidine following the administration of single doses to normal subjects

TABLE

NORMAL PATIENTS MEAN BLOOD CONCENTRATIONS AND URINE RECOVERIES WITH SINGLE DOSES OF SULPHADIMETHOXYPYRIMIDINE

All readings are means for two patients (50 g dose, one patient only)

	Mean blood concentrations of free and, in parentheses, conjugated drug in mg /100 ml									free a paren conju	urine eries of ind, in theses, igated ug	
Dose g	1 hr	2 hrs	3 hrs	6 hrs	12 hrs	24 hrs	2 days	3 days	4 days	5 days	mg	Per cent of dose
0.5	1 25	2 05	2 45	1 7	1 75	12	Trace				74	14 8
	(0 1)	(0 05)	(0 2)	(0 05)	(1 25)		(0 85)	T			(18) 218	(3 6) 21 8
10	1 4 (0 1)	2 15 (0 15)	2 65 (0 05)	2 65 (0 25)	3 1 (0 2)	(0)	(04)	Trace (1 6)			(46)	(5 3)
15	04	1 85	3 5	4 2	`5 65	5 05	42	2 9	2 05	19	500	33 3
	(0)	(1 05)	(1 0)	(0 95)	(0.7)	(0.85)	(0.7)	(0 2)	(0 35)	(0)	(77)	(4 4) 31 8
20	1 85	3 15 (0)	(0)	5 85	52	(0 35)	19 (02)	1 2 (0 25)	(Trace)	İ	637 (252)	(12.6)
40	1 15	4 05	60	8 75	10 05	8 45	67	4 55	3 65	2 85	835	30 6
	(0 25)	(01)	(0 25)	(0 65)	(0 45)		(0 4)	(0 45)	(0 25)	(0 2)	(241)	
5 0	3 3	50	69	71 (01)	8 8 (0 2)	8 7 (0 3)	5 8 (0)	47	3.0	(0.5)	1202 (415)	24 0 (8 3)
	(0)	(0)	(0 3)	(01)	(0 2)	(0.3)	(0)	(0)	(0)	(0.5)	(+13)	(0.5)

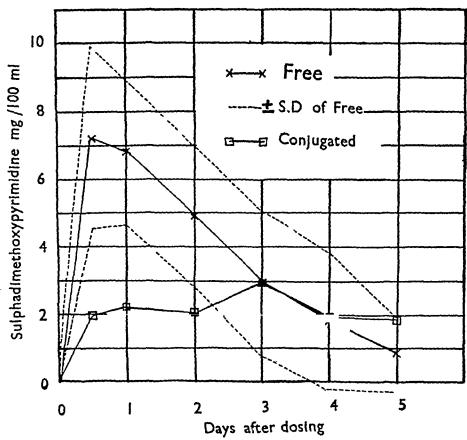


Fig 2—Blood concentrations of sulphadimethoxypyrimidine following the administration of a single dose of 5 g to pneumonia patients

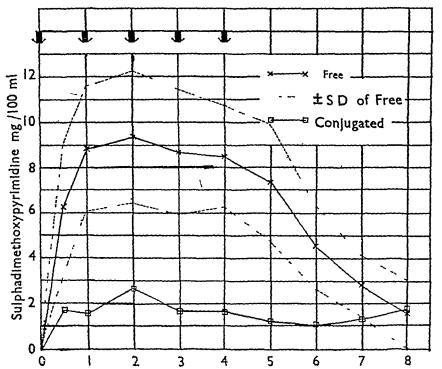


Fig 3—Blood concentrations of sulphadimethoxypyrimidine following the administration of an initial dose of 5 g, and then 3 g, daily to pneumonia patients

attained at three hours after a dose of 0.5 g, but with higher dosage the maximum was at 12 hours. After a single dose of 4 g a blood concentration of 10.05 mg/100 ml was attained after 12 hours and 2.85 mg/100 ml persisted five days after the dose, a similar dose of sulphamezathine gave a maximum of 13.2 mg/100 ml, falling to traces within 24 hours. For patients suffering from pneumonia a single dose of sulphadimethoxypyrimidine gave on the average lower maxima and less well sustained blood concentrations (Fig. 2). The average maximum attained after 5 g was 8.86 mg/100 ml, and only traces were detected in the blood after four days, although 2 mg/100 ml were still present after three days.

In patients treated with 5 g followed by 3 g every 24 hours, blood concentrations were usually well maintained (Fig 3) In a few cases, however, the drug concentration did not reach a level of 8 mg/100 ml, a concentration usually regarded as effective chemotherapeutically Patients with a low concentration of free drug usually showed a high percentage of acetylation, a frequent finding with other sulphonamides

Since clinical results were not completely satisfactory it was decided to try a dose of 5 g followed by 3 g every 12 hours (Fig 4) This dosage gave

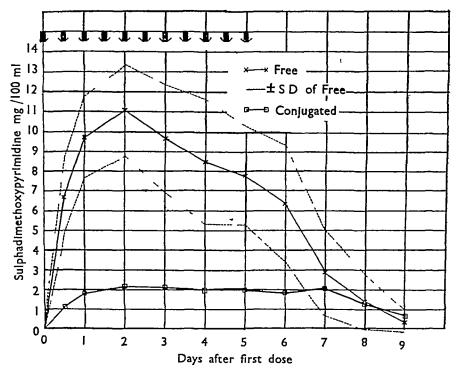


Fig 4—Blood concentrations of sulphadimethoxypyrimidine following the administration of an initial dose of 5 g., and then 3 g, at twelve-hourly intervals, to pneumonia patients

slightly higher blood concentrations. All patients (except one who had only three doses) attained blood concentrations of over 8 mg/100 ml. In nearly all pneumonia patients receiving the drug, whether as single or repeated doses, free sulphadimethoxypyrimidine was still present in appreciable quantities three days after the last dose. In one patient with staphylococcal pneumonia and considerable renal damage (blood urea 100 to 150 mg/100 ml), sulphonamide was still present in measurable quantities 16 days after the last dose, though the blood urea had fallen slightly

Samples of cerebrospinal fluid were obtained from two patients and contained sulphadimethoxypyrimidine concentrations, in one case of 58 per cent, and in the other of 31 per cent, of the simultaneous blood concentration

The blood concentration of sulphadimethoxypyrimidine reached a slightly lower maximum than did sulphamezathine after similar doses, but this maximum was attained later and high blood concentrations persisted for a very much longer period. There was a tendency for the concentrations reached in the controls to be higher than those attained with similar doses of the drug in febrile patients. Persistence of the blood concentrations after the last dose was definitely less in the febrile patients.

The recovery of the drug in the urine was comparatively low. The average recovery was 32 9 per cent, 26 6 per cent being free and 6 6 per cent conjugated in the normal patients, as compared with 86 2 per cent for sulphamezathine (Clark et al., 1943), 68 per cent for sulphadiazine (Reinhold et al., 1941), and 57 3 per cent for sulphapyridine (Long and Feinstone, 1938). The percentage of the drug recovered as acetyl compound, both from blood and urine, was low. These results must in part be interpreted in the light of the results of Gage et al. (1947) who record only partial recovery of the drug after acid hydrolysis.

Crystals of the drug were seen in many of the urines passed, but no renal symptoms were observed directly referable to the presence of the crystals. In two patients with severe renal damage this damage could not be definitely assigned to the effect of sulphadimethoxypyrimidine. The crystals took the form of St. Andrew's crosses and were shown chemically to be free sulphadimethoxypyrimidine. When compared with the needle-shaped crystals often observed in the urine of patients receiving other sulphonamides, sulphadimethoxypyrimidine would not be expected to cause such extensive mechanical injury to the renal structures. Confirmatory evidence was provided by estimation of blood urea in 34 patients. The blood urea was usually about 40 mg/100 ml on admission and in most cases tended to fall as the pneumonic condition improved. In no case was there a significant rise except in one woman dying within 48 hours of admission who was shown at autopsy to have severe chronic nephritis.

In chronic toxicity tests by Gage et al (1947) large doses of sulphadimethoxypyrimidine in rats gave rise to severe central necrosis of the liver lobules, and it serum bilirubin, protein, and phosphatase were therefore made on nine patients There was no change in the concentrations of these substances such as would indicate liver damage. It is realized that this series of tests would only show gross liver damage, and that minor impairment of function might pass unnoticed

SUMMARY

- 1 Sulphadimethoxypyrimidine in man persists in the blood stream longer than do the other common sulphonamides after similar doses
 - 2 Acetylation of the drug is slight
- 3 Single daily doses of sulphadimethoxypyrimidine will give blood concentrations of an order considered adequate for therapy in pneumonia

CLINICAL TRIAL

Sulphadimethoxypyrimidine was given to 41 patients with pneumococcal lobar pneumonia, whose ages varied between 16 and 78 years

An initial dose of 3 to 5 g, followed at intervals of 24 hours by two doses of 2 g, was found to give a blood level of 5-13 mg per 100 ml which was maintained for 40 to 60 hours Eighteen patients were treated in this way

Nine patients were given an initial dose of 5 g, followed by 3 g at intervals of 24 hours for four doses, a blood concentration of 5 to 14 mg per 100 ml being maintained for about 120 hours

In thirteen patients an initial dose of 5 g was followed at 12-hour intervals by two doses of 3 g and then by 2 g doses for several days, maintaining blood concentrations between 7-16 mg per 100 ml for four to six days. This method proved the best for maintaining adequate blood levels

The drug was well tolerated and showed quite definite therapeutic value, but was not so effective as other sulphonamides used in series of similar cases (Don et al., 1940, Macartney et al., 1942, Ramsay et al., 1945)

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AN ASSESSMENT OF THE VALUE OF SUGGESTED THERAPIES FOR LEUCOPENIA

BY

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(Received January 20, 1947)

The subcutaneous injection of methyl-bis(β -chloroethyl)amine (a nitrogen mustard) into rabbits consistently produces a leucopenia, and the degree of this leucopenia is readily varied by variation in the dosage of methyl-bis(β -chloroethyl)amine. This phenomenon has been used as a method of assessment of the value of three suggested therapies for leucopenia. The therapies tested were

- (1) Twenty-five per cent p-chloroxylenol in methylacetamide (CXM), which Zondek and Bromberg (1943) claim produces a leucocytosis lasting about four days in normal human subjects. In patients with typhoid fever the white cell count was maintained at a normal level by intramuscular injections of CXM, the count falling within 36 hours of discontinuing the injection
- (ii) The leucocytosis promoting factor (LPF) of Menkin and Kadish (1943) The latter state that 100 mg of this preparation in saline or phosphate buffer at pH 74 when given to animals either by cardiac puncture or by subcutaneous injection causes leucocytosis
- (iii) 1 g/100 c c aqueous sodium succinate, 01 c c of which, when injected subcutaneously into normal subjects or phthisic patients, is said to raise the white cell count (Hammett, Vessler, and Browning, 1917)

METHOD OF ASSAY

The leucopenia was produced by the subcutaneous injection of methyl-bis(β -chloroethyl)-amine hydrochloride solutions (1 mg/cc) Rabbits (circa 2 kg) and occasionally goats (circa 30 kg) were used as the test animals The therapies under test were given at various intervals after the methyl-bis(β -chloroethyl)amine hydrochloride injections, the efficacy of the therapies being judged by daily white cell counts performed between 10 a.m and noon and before feeding

RESULTS

(1) CXM—This was given by intramuscular injection. Zondek and Bromberg's dosage for man (i.e., two doses of 10 c.c. the first day and 10 c.c. daily for the next three days) was given to goats. In normal goats only a slight rise in the white cell count was produced, while in methyl-bis(β -chloroethyl)amine hydrochloride-poisoned animals leucopenia was not prevented and the mortality was actually doubled (Table I)

A similar picture was obtained with rabbits. In normal rabbits a slight leucocytosis was produced with small doses, but raising the dosage caused all

TABLE I EFFECT OF INTRAMUSCULAR CXM ON THE WHITE BLOOD CELL COUNTS OF GOATS

Number Dosage nitrogen animals mustard		Therapy dosage	Average white cell counts in thousands/cu mm on days						
ammais	mustaru	-	Z-1	Z	1	2	3	4	5
8		20 cc day Z 10 cc days 2 and 3	70	70	96	10 4	10 1	8 7	77
4	1 mg /kg day Z		99	11 0	100	3 0*	3 7	3 7	2 4*
4	1 mg /kg day Z	20 cc day 1 10 cc days 3 and 4		11 0	54	7 8*	4 6	2 9*	**

^{* -} one animal dead

the rabbits to die With methyl-bis $(\beta$ -chloroethyl)amine-poisoned rabbits leucopenia was not prevented and the mortality was increased (Table II)

TABLE II

EFFECT OF INTRAMUSCULAR CXM ON THE WHITE BLOOD CELL COUNTS OF RABBITS

Number of animals	Dosage nitrogen mustard	Therapy dosage	Average white cell counts in thousands/cu mm on days								
annnais	mustard	}	Z-1	Z	1	2	3	4	5	6	8
4	_	05cc/kg day Z 1cc/kg day 1	92	93	12 3	12 2	93*	63			
3	-	50cc/kg day Z	79	71	69*	6 2*	3 0*	1			
4	1 mg/kg day Z	1 cc/kg day 1 0 5 cc/kg day 2		8 4	67	6 2*	12	2 4*	29	74	
5	1 mg/kg day Z	5 cc/kg day 1		74	8 1	70	***				-
5	1 mg/kg day Z	2 cc/kg day 1 1 cc/kg day 2	74	7 5	74	68	6 5	4 4**	3 1	3 6**	
5	1 mg/kg day Z	1 cc/kg days 1 and 2	6 5	70	65	64	5 8	49	3 4	3 4*	3 8
10	1 mg /kg day Z	_	10 4	11 5	93	77*	6.3	5 4	4 8	8 3	92

 ⁼ one animal dead

(11) LPF—This was prepared from the inflammatory pleural exudates (produced by injection of turpentine) of rabbits and goats (LPF(R) and LPF(G)) When injected subcutaneously into normal rabbits or goats there was some evidence of the production of a slight leucocytosis, but with methyl-bis(β -chloro-

ethyl)amine hydrochloride-poisoned animals the leucopenia was not prevented and the death-rate was actually increased (Table III)

TABLE III -EFFECT OF LPF ON THE WHITE BLOOD CELL COUNTS OF GOATS AND RABBITS

Number	Dosage nitrogen	Therapy and dosage	Average white cell counts in thousands/cu mm on days								
anımals	mustard		Z-1	Z	1	2	3	4	5	6	7
(a) RABBI 5	TS	100 mg LPF(R) day Z and 10 mg day 1	10 5	11 0	11 0	96	10 0	10 4			
4	-	100 mg LPF(G) day Z	70	68	91	11 3	89	13 2	14 9	11 6	12 3
5	1 mg /kg day Z	100 mg LPF(R) day	96	97	96	4 8	4 6*	8 4**	8 2	10 9	10 0
3	1 mg/kg day Z	100 mg LPF(R) day 1 and 2		81	-87	59	12	13	5 0**	10 2	
5	1 mg/kg	100 mg LPF(G) day	91	10 2	96	89	9 2**	98	13 0*	98	86
4	day Z 1 mg /kg day Z	100 mg LPF(G) day 1 and 2	-	75	70	39	1 0*	0 5	12	53	
10	1 mg /kg day Z	~_	10 4	11 5	93	77*	63	5 4	48	83	92
(b) GOAT	S .	200 I DE(C) d-	7.2	F. C	12.0	11 5	10.0	0.0	0.0	0.4	
2	-	300 mg LPF(G) day Z and 1	ł	56		11 5	108	90	98	8 4	
6	1 mg /kg day Z	300 mg LPF(G) day 1 and 2	8 1	79	14 9	99	73	5 5**	5 8**	5 4	
4	1 mg /kg day Z	_	99	11 0	10 0	3 0*	3 7	3 7	2 4*	25	61

^{* =} one animal dead

(111) Sodium succinate — Various quantities and concentrations of sodium succinate in aqueous solution were injected subcutaneously into normal rabbits

TABLE IV EFFECT OF SODIUM SUCCINATE ON THE WHITE BLOOD CELL COUNTS OF NORMAL RABBITS

Number of animals	Therapy dosage	Average white cell counts in thousands/cu mm on days								
aiiiiiais	_	Z-1	Z	1	2	3	4	5	6	7
5 5 5 5	01 cc 1% solution day Z 05 cc 1% solution day Z 1 cc 1% solution day Z 1 cc 1% day Z, twice daily, days 1, 2, 3 1 cc 10% solution day Z	86 92 98 91	89 96 81 98 155	10 9 11 9 11 1 11 8	11 9 13 1 11 9 14 2 12 4	12 0 12 1 12 0 20 8	93 95 131 108	9 2 9 2 9 2 14 2 8 9	15 0 11 9	98

and there was definite evidence of the production of a leucocytosis, a 1 g/100 c c solution being better than a 10 g/100 c c concentration (Table IV) However, the sodium succinate solutions did not prevent the occurrence of leucopenia in methyl-bis(β -chloroethyl)amine hydrochloride-poisoned animals (Table V)

TABLE V

EFFECT OF SODIUM SUCCINATE ON THE WHITE BLOOD CELL COUNTS OF
NITROGEN MUSTARD POISONED RABBITS

Number of animals	Dosage nitrogen mustard	Therapy dosage				erage v usands				•	
	Musiaid		Z-1	Z	1	2	3	4	5	6	7
10	1 mg /kg day Z		10 4	11 5	93	77*	63	54	48	8 3	9.2
15	1 mg /kg day Z	1 c c 1% day 1	96	94	11 4	10 2	57	60*	10 2	10 5	
5	1 mg /kg day Z	1 c c 1% at Z + 6 hours	<u> </u>	12 0	13 9	10 2	69	66	12 7	-	-
10	1 mg /kg day Z	05 cc 1% day 1	96	10 3	10 7	11 5	10 0*	11 3	118	12 0	10 6
5	1 mg /kg day Z	1 c c 10% day 1	8 7	90	4 5	42	57	3 3**	-	_	_
10	2 mg /kg day Z	_	10 5	14 0	12 5*	11 1*	92	3 9	11 7* *	11 5	15 3
5	2 mg /kg	1 c c 1% days 1, 2, 3 and 4	13 2	13 6	14 9	167	67	10 4	16 8	12 9	
10	day Z 2 mg /kg day Z	1 cc 1% days 1-6	9 5	12 7	89*	69	51	2 7**	13 4*	119	79
5	2 mg /kg day Z	5 c c 10% day 1	8 4	10 1	8 8	38	04	03*	3 7		
5	2 mg./kg day Z	1 cc 1% at Z + 6 hours	92	8 4	14 5	42	09*	3 5**			

^{* -} one animal dead

SUMMARY

When assayed on rabbits or goats poisoned with methyl-bis(β -chloroethyl)-amine hydrochloride (given by subcutaneous injection), the following substances were ineffective in preventing leucopenia

- 1 p-chloroxylenol in methylacetamide—this preparation, indeed, was found to be toxic in the recommended dosage
 - 2 The leucocytosis-promoting factor of Menkin
 - 3 An aqueous solution of sodium succinate

I am indebted to the Chief Scientific Officer, Ministry of Supply, for permission to publish this paper

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SELECTIVE INHIBITION OF PSEUDO-CHOLINESTERASE BY DIISOPROPYL FLUOROPHOSPHONATE

ΒŸ

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The inhibition of cholinesterase by fluorophosphonates was discovered in 1941 by Adrian, Feldberg, and Kilby (1947), when they examined dimethyl In 1941 McCombie and Saunders prepared disopropyl fluorophosphonate fluorophosphonate and Adrian et al (1942), as well as Mackworth (1942) found that it had an even stronger inhibitory action on cholinesterase than the dimethyl ester At that time it was not known that there were two enzymes, true cholinesterase and pseudo-cholinesterase (Mendel and Rudney, 1943a), which are not necessarily affected similarly by inhibitors (Mendel and Rudney, 1944, Hawkins and Gunter, 1946) In the experiments to be reported in this paper it will be shown that disopropyl fluorophosphonate, unlike eserine or prostigmine (Hawkins and Mendel, 1946, and unpublished experiments). exhibits a much stronger inhibitory action on pseudo-cholinesterase than on true With low concentrations of disopropyl fluorophosphonate it is therefore possible to inhibit pseudo-cholinesterase selectively without affecting true cholinesterase

The possibility of such selective inhibition of pseudo-cholinesterase by dissopropyl fluorophosphonate was suggested by the following two observations

(1) Bodansky (1945) as well as Mazur and Bodansky (1946) found that on exposure of human beings to low concentrations of the vapour of dissopropyl fluorophosphonate almost complete inhibition of cholinesterase activity in the plasma could be obtained without causing serious distress. It should be borne in mind, however, that human plasma contains predominantly pseudo-cholinesterase (Mendel, Mundell, and Rudney, 1943) and that the inhibition of this enzyme, as shown by Hawkins and Gunter (1946), will not interfere with the destruction of acetylcholine released by nervous activity. These workers found that certain concentrations of a prostigmine analogue, the dimethylcarbamate of 2-hydroxy-5-phenyl-benzyltrimethylammonium bromide (Hoffman-LaRoche Nu-683), are capable of inhibiting completely the activity of pseudo-cholinesterase in vitro without affecting significantly that of true cholinesterase. This

compound, when injected into dogs in amounts sufficient to inhibit pseudo-cholinesterase almost completely, elicits no symptoms indicative of the accumulation of acetylcholine, such symptoms appear only if the dose injected is sufficiently large to depress the activity of the true cholinesterase as well Pseudo-cholinesterase, therefore, plays no essential role in the hydrolysis of acetylcholine in vivo

(2) Bodansky and Mazur (1946) and Mazur and Bodansky (1946) found that the concentration of disopropyl fluorophosphonate necessary for the inhibition of cholinesterase varied according to the enzyme preparations used, the negative logarithm of the molar concentration of disopropyl fluorophosphonate necessary to produce a 50 per cent inhibition of the activity towards acetylcholine $(1.5 \times 10^{2} M)$ was 7.7 and 8.1 for human and horse serum respectively, the corresponding value for rabbit serum was 4.1, and the values for red blood cells and brain varied between 5.2 and 6.0. Since the sera of man and the horse contain predominantly pseudo-cholinesterase (Mendel, Mundell, and Rudney, 1943), rabbit serum mainly true cholinesterase (Mendel and Rudney, 1945), and brain and red blood cells throughout the animal kingdom true cholinesterase only (Mendel and Rudney, 1943a, 1943b), the results obtained by Mazur and Bodansky can be interpreted as indicating that pseudo-cholinesterase is approximately 100 times more sensitive to the inhibitory action of disopropyl fluorophosphonate than is true cholinesterase

METHODS

Cholinesterase activity was measured manometrically by Warburg's method at 37° C in $2.5 \times 10^{-3} M$ NaHCO, saturated with 5 per cent CO, in N. The dissopropyl fluorophosphonate was added to the bicarbonate medium containing the enzyme preparation in the main compartment of the Warburg flask, the substrate being placed in the side arm After the enzyme preparation had been shaken for 15 min to attain temperature equilibrium the substrate was tipped into the main compartment. From a stock solution, freshly prepared every third day, of dissopropyl fluorophosphonate ($10^{-3}M$) in propylene glycol, greater dilutions were made with distilled water as required. The final concentration of propylene glycol present in the experimental vessels caused by itself no inhibition of the cholinesterases

The activities of the true and pseudo-cholinesterases were measured, as described by Mendel, Mundell, and Rudney (1943), by the rates of hydrolysis of acetyl- β -methylcholine and benzoylcholine respectively

RESULTS

In vitro—Preliminary experiments were carried out to determine the inhibitory action of disopropyl fluorophosphonate on enzyme preparations containing either only true cholinesterase or only pseudo-cholinesterase. This was done in order to ascertain whether the substrate acetylcholine could be replaced by acetyl-β-methylcholine in measuring the activity of true cholinesterase or by benzoylcholine in measuring the activity of pseudo-cholinesterase, without affecting the percentage inhibition by disopropyl fluorophosphonate Haemolysed dog erythrocytes were used as a source of true cholinesterase, and an extract of dog pancreas as a source of pseudo-cholinesterase. As shown in Table I, the degree of

inhibition of true cholinesterase and pseudo-cholinesterase by dissopropyl fluorophosphonate is not altered when acetyl- β -methylcholine or benzoylcholine respectively are substituted for acetylcholine

TABLE I
INHIBITION OF PSEUDO-CHOLINESTERASE AND TRUE CHOLINESTERASE BY DIISOPROPYL
FLUOROPHOSPHONATE, USING VARIOUS SUBSTRATES

Enzyme preparation	Substrate*	Molar concentration of diisopropyl fluorophosphonate	Percentage inhibition of enzymatic activity
True cholinesterase (haemolysed dog erythrocytes)	Ach $12 \times 10^{-3}M$ Mch $3 \times 10^{-2}M$ Ach $12 \times 10^{-3}M$ Mch $3 \times 10^{-2}M$	1 × 10 ⁷ 1 × 10 ⁷ 5 × 10 ⁷ 5 × 10 ⁷	4 3 25 26
Pseudo-cholinesterase (suspension of dog pancreas)	Ach $6 \times 10^{-2}M$ Bch $6 \times 10^{-3}M$ Ach $6 \times 10^{-2}M$ Bch $6 \times 10^{-3}M$	1 × 10 8 1 × 10 8 5 × 10-8 5 × 10-8	87 86 100 100 ~

^{*} Ach = acetylcholine, Mch = acetyl-β-methylcholine, Bch /= benzoylcholine

Moreover, the presence of pseudo-cholinesterase does not interfere with the inhibition of the true cholinesterase. Table II shows that the hydrolysis of acetyl- β -methylcholine by the true cholinesterase of haemolysed human erythrocytes is inhibited 26 per cent by $5 \times 10^{-8} M$ dissopropyl fluorophosphonate. When highly purified pseudo-cholinesterase prepared from horse serum is mixed with the haemolysed erythrocytes in an amount possessing an activity approximating that of the pseudo-cholinesterase of human plasma, no diminution of the inhibitory action of dissopropyl fluorophosphonate on true cholinesterase is observed

In subsequent experiments, therefore, acetyl- β -methylcholine and benzoylcholine could be used to estimate separately the activities of the two cholinesterases in enzyme preparations which in most cases contained a mixture of both

TABLE II
INHIBITORY ACTION OF DIISOPROPYL FLUOROPHOSPHONATE ON TRUE CHOLINESTERASE IN
THE PRESENCE OF PSEUDO-CHOLINESTERASE

Type of cholinesterase	Molar concentration of dusopropyl fluorophos- phonate	Activity* (με CO ₂ /15 min)	Percentage Inhibition
True cholinesterase (haemolysed human erythrocytes)		100 0	_
True cholinesterase (haemolysed human erythrocytes) True cholinesterase (haemolysed human	5 × 10 ⁻⁸	74 0	26 0
erythrocytes) in the presence of pseudo- cholinesterase† (purified horse serum)	5 × 10 ⁸	74 5	25 5

^{*} Substrate in all cases acetyl- β -methylcholine (3 × 10⁻²M)

[†] The pseudo-cholinesterase from horse serum was kindly supplied by Miss F Strelitz, who purified it according to her method (Strelitz, 1944) This preparation exhibited no activity towards acetyl-\(\beta\)-methylcholine

The enzyme preparations tested were the plasma of man, dog, cat, rat, rabbit, and sheep Sheep plasma contains no pseudo-cholinesterase, while the plasma of the other species contains both cholinesterases, although in different proportions. For each enzyme preparation, with the exception of sheep plasma, the minimal concentration of disopropyl fluorophosphonate required to cause complete inhibition of pseudo-cholinesterase activity was determined, using benzoylcholine as substrate. The inhibitory action of this concentration of disopropyl fluorophosphonate on the true cholinesterase in the plasma was then examined, using acetyl-β-methylcholine as substrate.

The results of these experiments are shown in Table III Although pseudo-cholinesterase is inhibited completely in all instances, the true cholinesterase is inhibited only partially 35, 34, and 33 per cent in human, rabbit, and rat plasma respectively, and only 7 per cent in dog plasma

TABLE III
SELECTIVE INHIBITION OF PSEUDO-CHOLINESTERASE BY DIISOPROPYL FLUOROPHOSPHONATE

Source of enzyme	Substrate*	Molar concentration of dusopropyl fluorophosphonate	as μ l CC by 1 ml	(expressed 2 evolved plasma min) With inhibitor	Percentage inhibition
Human	Ach $6 \times 10^{-2}M$	1 × 10 ⁻⁸	1280	13	99
plasma	Mch $3 \times 10^{-2}M$,,	26	17	35
	Bch $6 \times 10^{-3}M$		570	0	100
Dog	Ach $6 \times 10^{-2}M$	5 × 10-8	590	60	90
plasma	Mch $3 \times 10^{-2}M$,,	82	76	7
~ .	Bch $6 \times 10^{-3}M$	- ',	294	0	100
Cat	Ach $6 \times 10^{-2}M$	5 × 10-8	426	14	96
plasma	Mch $3 \times 10^{-2}M$,,	30	25	16
D - 4	Bch $6 \times 10^{-3}M$	1 × 10-8	109	0	100
Rat	Ach $6 \times 10^{-2}M$	1 × 10-°	123	16	87
plasma	Mch $3 \times 10^{-2}M$,,	54	36	33
Rabbit	Bch $6 \times 10^{-3}M$ Ach $6 \times 10^{-2}M$	5 × 10-7	20	0	100
plasma	Ach $6 \times 10^{-2}M$ Mch $3 \times 10^{-2}M$	3 × 10 ·	45 47	20 31	,56 34
piasilia	Bch $6 \times 10^{-3}M$,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,		0	100
Sheep	Ach $6 \times 10^{-2}M$	5 × 10-7	4 13	10	23
plasma	Mch $3 \times 10^{-2}M$	1	14	11	22
promin	Bch $6 \times 10^{-3}M$	"	17	Ô	
	25 0 1 25 1	,,			

*Ach = acetylcholine
Mch = acetyl-\(\beta\)-methylcholine
Bch = benzoylcholine

*Ach | acetylcholine
Ball in the form of the chloride
Bch | benzoylcholine

Besides disclosing the difference between the sensitivities of pseudo-cholinesterase and true cholinesterase towards dissopropyl fluorophosphonate, these experiments show that when acetylcholine serves as substrate, the inhibition brought about by dissopropyl fluorophosphonate depends on the proportion of true cholinesterase and pseudo-cholinesterase present in the plasma, the greater the content of pseudo-cholinesterase, the greater the discrepancy between the inhibition of true cholinesterase and the inhibition observed when acetylcholine

is the substrate, conversely, the lower the pseudo-cholinesterase activity, the closer the parallelism between the inhibition of true cholinesterase and the inhibition of the acetylcholine hydrolysis

In vivo -Mazur and Bodansky found that in human beings exposed to disopropyl fluorophosphonate, an inhibition of 98-99 per cent of the activity of the plasma towards acetylcholine did not result in symptoms of acetylcholine accumulation series, animals were injected intramuscularly with dissopropyl fluorophosphonate in order to ascertain whether in species, the plasma of which contains true cholinesterase and pseudocholinesterase in a proportion different from that in human plasma, there is also no correlation between the inhibition of the activity of the plasma towards acetylcholine and the symptoms to be expected from this inhibition

Rabbits were chosen as experimental animals because the plasma of this species contains a smaller proportion of pseudo-cholinesterase to true cholinesterase, and therefore (see Table III) the discrepancy between the degree of inhibition of the activity towards acetylcholine, on the one hand, and towards acetyl- β -methylcholine, on the other, is not so pronounced as with human plasma, in which pseudo-cholinesterase predominates

It will be seen from the typical experiment outlined in Table IV that rabbits receiving intramuscular injections of disopropyl fluorophosphonate display their first symptoms of acetylcholine poisoning (i.e., masticatory movements of the laws and slight generalized fibrillation) at a time when an appreciable activity (18 per cent) of the plasma towards acetylcholine is still present. These results confirm Mazur and Bodansky's findings in their experiments with rabbits ever, these authors did not attempt to explain why in rabbits symptoms of acetylcholine poisoning appear when the cholinesterase of their serum still displays a considerable activity towards acetylcholine, whereas in man an almost complete inhibition of the activity of the plasma towards acetylcholine causes no symptoms of serious distress

TABLE IV

RELATIONSHIP BETWEEN THE INHIBITION OF CHOLINESTERASES BY DISOPROPYL FLUORO-PHOSPHONATE AND THE ONSET OF SYMPTOMS OF ACETYLCHOLINE POISONING

Rabbit II—2 5 kg 11 26 Activity of plasma tested

11 27 0 65 mg disopropyl fluorophosphonate* in saline injected intramuscularly localized twitching of hind leg at site of injection

11 29 11 40

masticatory movements, which continued until 0 13 mg dissopropyl fluorophosphonate in saline intramuscularly 11 49

generalized fibrillation 11 56

11 57 chewing, swallowing and fibrillation, activity of plasma tested

Time	Activity (expressed as μ ¹ CO ₂ evolved by 1 ml plasma in 15 min) towards					
	Bch † (6 × 10 ⁻³ M)	Inhibition %		Inhibition %	Ach † (6 × 10 ⁻² M)	Inhibition %
11 26 11 57	6 1 0	100	62 8 17 3	73	69 5 13 3	82

^{*} An initial dilution (1 in 500) was made with propylene glycol

[†] Bch = benzoylcholine, Mch = acetyl- β -methylcholine, Ach = acetylcholine

DISCUSSION

The plasma of most species contains, in varying proportions, a mixture of two enzymes pseudo-cholinesterase, which plays no essential role in the hydrolysis of acetylcholine in vivo, and true cholinesterase, the inhibition of which results in symptoms of acetylcholine poisoning. The experiments reported here have shown that appropriate concentrations of disopropyl fluorophosphonate completely inhibit pseudo-cholinesterase without affecting the true cholinesterase significantly (see Table III)

Since acetylchòline is hydrolysed by both cholinesterases, measurements with acetylcholine as substrate can yield no information about the contribution made by each of these enzymes to the total activity, and the extent of inhibition of the activity towards acetylcholine in the presence of a selective inhibitor of pseudo-cholinesterase will depend on the relative proportions of pseudo- and true cholinesterases in the mixture which is being tested. Therefore, the degree of inhibition of acetylcholine hydrolysis by dissopropyl fluorophosphonate is no index of the inhibition of the true cholinesterase.

In the light of the above facts it is not surprising that human beings exposed to low concentrations of disopropyl fluorophosphonate exhibit no symptoms indicative of acetylcholine accumulation when their plasma has lost 98-99 per cent of its original activity towards acetylcholine, 99 per cent of the activity of human plasma towards acetylcholine ($6 \times 10^{2}M$) is due to pseudo-cholinesterase, true cholinesterase accounting only for about 1 per cent of the total activity (Mendel, Mundell, and Rudney, 1943) Consequently, when disopropyl fluorophosphonate causes a 98-99 per cent inhibition of the activity of human plasma towards acetylcholine, the inhibition of the pseudo-cholinesterase activity should be complete, while the activity of the true cholinesterase may be depressed less than 35 per cent (see Table III)

The results are entirely different with animals whose plasma contains predominantly true cholinesterase (e.g., rabbits). The hydrolysis of acetylcholine by the plasma of such animals is due mainly to the true cholinesterase, therefore, when a 98 per cent inhibition of the activity of their plasma towards acetylcholine is achieved, it must be the true cholinesterase which is inhibited to a great extent. Consequently, symptoms of acetylcholine poisoning should set in at a much lower level of inhibition of acetylcholine hydrolysis than would be the case in species, such as man, where the hydrolysis of acetylcholine by the plasma is due mainly to pseudo-cholinesterase. Indeed, our experiments with rabbits have shown that the injection of disopropyl fluorophosphonate leads to parasympathomimetic symptoms and fibrillation at a time when the activity of the plasma towards acetylcholine is inhibited not more than 80–82 per cent. In sheep, whose plasma contains true cholinesterase only, these symptoms would probably appear at a still lower level of inhibition of the acetylcholine hydrolysis. It would seem, therefore, that the higher the ratio of true cholinesterase to

pseudo-cholinesterase, the lower the degree of inhibition of acetylcholine hydrolysis prevailing at the time of onset of symptoms

As mentioned previously, it is the inhibition of true cholinesterase which results in the appearance of symptoms of acetylcholine poisoning On the basis of experiments in which the level of true cholinesterase activity was correlated with the appearance of symptoms after the injection of eserine, Gunter and Mendel (1945) concluded that the body possesses a surplus of this enzyme, they observed no ill-effects until the activity of the true cholinesterase was inhibited Similarly, Hawkins and Gunter (1946) found that symptoms of acetylcholine accumulation made their first appearance in dogs when the true cholinesterase activity of their plasma had been depressed to 23 per cent of its Koelle and Gilman (1946) reported only slight parasympathooriginal level mimetic symptoms in rats when the activity of the true cholinesterase in the brain had been depressed to 21-28 per cent of the normal by intramuscular injection of dusopropyl fluorophosphonate, and in the present study symptoms of acetylcholine accumulation appeared in rabbits when the activity of the true cholinesterase in the plasma had been depressed to 27 per cent of its original level (see Table IV)

Therefore, disopropyl fluorophosphonate, in order to produce symptoms of acetylcholine poisoning, must be present in a concentration which is sufficient to remove the true cholinesterase in excess of that required for normal function To estimate to what extent this objective has been achieved by injection of, or exposure to, disopropyl fluorophosphonate it is necessary to determine the degree of inhibition of the activity towards acetyl- β -methylcholine. The use of acetylcholine as substrate would yield no such information except in the rare cases in which pseudo-cholinesterase is absent or is present in negligible amounts only

SUMMARY

- I Although dissopropyl fluorophosphonate inhibits both true and pseudo-cholinesterases, higher concentrations are required for the inhibition of true cholinesterase than of pseudo-cholinesterase. With appropriate concentrations of dissopropyl fluorophosphonate it is therefore possible, in a mixture of both enzymes, to inhibit selectively the activity of pseudo-cholinesterase without affecting that of true cholinesterase
- 2 Acetylcholine is hydrolysed *in vitro* not only by true cholinesterase, but also by pseudo-cholinesterase, therefore, measurements of cholinesterase activity in which acetylcholine is used as substrate cannot be used to correlate the degree of inhibition of true cholinesterase by dissopropyl fluorophosphonate and the effects resulting from this inhibition *in vivo*. Since true cholinesterase is the enzyme responsible for the hydrolysis of acetylcholine released at nerve endings, it is the degree of inhibition of true cholinesterase which must be determined

when a correlation between anti-cholinesterase action and pharmacological effects is sought

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A PRELIMINARY REPORT OF THE TOXICITY AND THE ASSOCIATED BLOOD CONCENTRATIONS OF PALUDRINE* IN LABORATORY ANIMALS

BY

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During the preparatory work before paludrine (4888) was administered to human beings the usual investigations were made of its toxicity in various laboratory animals. Early in the work we recognized that different species of animals behaved differently towards it, the rat and the mouse, for example, appeared more susceptible than the chick. We also recognized that the differences were possibly not due to a difference in susceptibility to unchanged drug, but more likely to a difference in its metabolism and the liberation of greater or less amounts of toxic by-products. Sufficient work was done to justify giving the drug to human beings, and the Liverpool workers (Adams, Maegraith, King, Townshend, Davey, and Havard, 1945) pursued their investigations on the assumption that human beings might react like the mouse and the rat, which are amongst the most susceptible of the laboratory animals. It was for this reason that, in the beginning, paludrine was given only twice daily, and doses were progressively increased by amounts not greater than 25 mg. Quite quickly it was shown that man must be classed amongst the least susceptible animals.

The laboratory results have not been published earlier because it was hoped that a fuller investigation would be made. Unfortunately, this will take longer than was planned, and because paludrine has now been sent to many laboratories in different parts of the world we are making the preliminary results available without further delay for the convenience of other workers

The constitution of paludrine (base) is given below

Two salts were used in the experiments, the monoacetate and the monohydrochloride The former contains 81 per cent by weight of the base and is soluble

^{*} Paludrine is the registered name for N₁-p-chlorophenyl-N₅-isopropylbiguanide

to the extent of about 2 per cent in water, the latter contains 87 4 per cent by weight of the base and is about half as soluble. Solutions of either salt are stable when boiled. The figures quoted in the text, unless it is stated to the contrary, refer to the salts. The monoacetate was used in the intravenous and intraperitoneal tests, the monohydrochloride in the oral tests.

Acute toxicity

This was measured in the usual way. Solutions were administered orally by means of a catheter tube, and intravenously or intraperitoneally by rapid (3 sec) injection. The results are given in Table I. For most species of animal three sets of figures are quoted which give, respectively, approximately the largest dose permitting all animals to live (LD0), the dose which kills approximately half the experimental animals (LD50) and approximately the smallest dose which kills all (LD100)

TABLE I ACUTE TOXICITY OF PALUDRINE IN LABORATORY ANIMALS

Species	Route	LD0 (mg/kg)	LD50 (mg/kg)	LD100 (mg /kg)
Chick (wt 50 g)	Oral 1 V	200 40	400–600 60–80	100
Mouse (wt 18-22 g)	Oral 1 v 1 p	50 10 10	60–80 20–30 20–30	100 40–50 40–50
Rat (wt 100 g)	Oral 1 y 1 p	80 20 20	100–150 40 40	60 60
Rabbit (wt 15 kg)	Oral 1 V	30	circa 150 cırca 50	

The intravenous or intraperitoneal injection of paludrine into both rats and mice is associated with delayed deaths, a point which is of much interest. It is best emphasized by comparing the results of an intravenous test using this drug with one using a closely related substance (4430), which differs only by a methyl group (see formula above) The results are given in Table II

- TABLE II

COMPARISON OF THE RESULTS FOLLOWING THE RAPID INTRAVENOUS INJECTION OF 4430 AND PALUDRINE INTO MICE

Dose	Results							
	4430	Paludrine						
100 mg /kg 80 mg /kg	5/6 mice dead within 3 min 5/6 mice dead within 3 min, survivor alive 5 days later	12/12 mice dead within 3 min 9/18 mice dead within 3 min, remaining 9 died 1 to 24 hours after the injection						
60 mg /kg	6/6 survived 5 days	No immediate deaths, 12/12 mice died 2 to 24 hours after the injection						

At the time these experiments were carried out the blood concentrations of paludrine had not been measured, and it was thought possible that the delayed deaths were caused by unusually prolonged retention of the drug in the blood, and therefore that an additive effect might be produced by further intravenous injections

The idea was tested by giving a second injection, after various intervals, of an amount (20 mg/kg) that, by itself, produced very few deaths. Seventy-two mice were injected in the beginning, 12 were kept as controls and the remainder were divided into five further groups of 12 which were given a second injection 1, 3, 6, 24, and 48 hours respectively, after the first injection. The results are given in Table III. (The results of a second, similar experiment are given in parentheses in the Table)

TABLE III

MORTALITY IN MICE AFTER A SECOND INTRAVENOUS INJECTION OF 20 MG /KG PALUDRINE
FOLLOWING A FIRST INJECTION OF THE SAME AMOUNT

Figures in parentheses are the results of a second experiment

Const			Total dead after			
	Group	0-1 hr	1–5 hr	5-24 hr	24-48 hr	5 days
I	Control				1/12 (1/12)	1/12 (1/12)
	2nd injection after					
III IV V VI	1 hr 3 hr 6 hr 24 hr 48 hr	1/12	1/12	9/12 9/12 11/12 (6/12) 4/12 (1/12)	2/12 	12/12 9/12 11/12 (6/12) 7/12 (3/12) 1/12

In mice, therefore, the second injection clearly exerts an additive effect Similarly, a second injection of paludrine into rats also produces an additive toxic effect (Table IV), and in them, too, its parenteral injection is associated with delayed deaths. On the other hand, in chicks which have received paludrine intravenously, deaths occur within about 15 min or not at all, and in them a second injection does not produce an additive effect

TABLE IV

MORTALITY IN RATS AFTER A SECOND INTRAVENOUS INJECTION OF 25 MG 'KG PALUDRINE FOLLOWING A FIRST INJECTION OF THE SAME AMOUNT

Group			Tatal				
	Group	0-1 hr	1-4 hr	4–8 hr	8-24 hr	24-48 hr	Total Deaths
I	Control				_		0/6
	2nd injection after	(
III	1 hr 3 hr (1/6 dead before 2nd injection)	2/6	_	1/6 2/5	3/6 2/5	_	6/6 4/5
IV V	6 hr 24 hr	_		2/6 —	3 6 —	_	5/6 0′6

Although these results tended to support the suggestion that paludrine might be highly persistent in rats and mice, measurements of blood concentrations soon disproved this, and we now find it difficult to believe that delayed deaths or additive effects are due to unchanged paludrine. We are therefore searching for a metabolite in the hope that the properties of the latter may provide an explanation

Chronic toxicity

(a) In mice—Two types of experiment were done. In the first, mice weighing 18 to 22 g were arranged in groups of 10 and dosed twice daily for 5 days with the test solutions. The LD50 in this experiment is about 25 mg/kg, at 12 5 mg/kg no animals die, at 50 mg/kg they all die

In the second type, young mice weighing 14 to 16 g were dosed twice daily for 14 days. Growth appeared normal amongst those receiving 12.5~mg/kg, deaths occurred at higher doses

(b) In rats —Newly weaned rats, weighing about 40 g, and selected from as few litters as possible, were arranged in groups of 10 Sexes and litter mates were distributed equally among the various groups Food (standard cubes made to a formula of the Rowett Institute) was given to the animals immediately after the daily weighing at 10 am, water was always available

The growth of rats for the first few weeks after weaning is linear and, with careful matching, all the groups in experiments such as the ones being described can be made to follow the same straight line. Seven days were allowed for the line to become established, and then treatment with paludrine was commenced. It was given orally, once daily

It was found that a dose of 50 mg/kg caused an immediate alteration in the slope of the growth curve, although not sufficient to reduce it to zero. Scattered deaths also occurred with this treatment. With a dose of 40 mg/kg a slight deviation of the curve was caused sometimes immediately, sometimes later. With a dose of 30 mg/kg, growth was normal over the whole period of treatment (two months in some experiments) and there were no deaths

Rats which died, and the survivors of all groups, were subjected to a pathological examination, but nothing of significance was found* It is noteworthy that in none of these toxicity tests has any symptom been produced in any of the mammalian species that would lead one to suppose that the drug had affected the central nervous system Chemical estimations confirm that the amount of drug which can be recovered from the brain of rats and rabbits is insignificant (see below and Spinks, 1947)

Blood concentrations†

The rat growth test just described is probably one of the most sensitive toxicity tests available in the laboratory and it was regarded as important to determine the concentrations of paludrine in the blood associated with the doses,

^{*}We are indebted to Dr J R M Innes for this information. The organs examined were brain, kidney, liver, pancreas, spleen, lung, intestine, and thyroid

[†] All concentrations, whether in plasma, whole blood or tissue, are given as mg/l or mg/kg of the free base

50 mg/kg and 30 mg/kg per day respectively, which delimited the toxic region. They were measured by the method of Spinks and Tottey (1946). Measurements on whole blood were made, in different experiments, after the first dose and after the seventh dose. At least three rats were used in the determination of each point. A curve for the concentrations reached on the seventh day is given in Fig. 1.

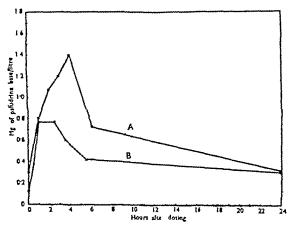
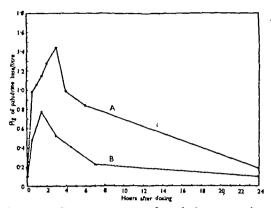


Fig 1—Concentrations of paludrine in the whole blood of rats after the seventh dose of 50 mg/kg (A) and 30 mg/kg (B) once daily

The most interesting feature of the results is the fact that the blood concentration associated with a dose of 50 mg/kg once daily, which produces deaths in some rats, is comparatively low and has a peak of only about 1 4 mg/l That such a concentration should be toxic in rats is of interest, because we believe it to be readily tolerated by human beings, 700 mg of paludrine have been administered twice daily in man with only mild toxic effects (Adams et al, 1945), while plasma concentra-

tions of about 0.5 mg/1 have been recorded twelve hours after doses of 500 mg twice daily (Maegraith et al, 1946) The maximal concentration in the plasma following the latter dose was found to be about 0.7 mg/l in one subject, who, however, showed minimal concentrations rather lower than normal (Maegraith Since the whole blood concentration in man is et al, private communication) between 2 and 3 times the plasma concentration (Maegraith et al., 1946), it is reasonable to assume that blood concentrations between 1 mg and 15 mg/l are attained following the administration of 500 mg twice daily The comparison can also be made on the basis of plasma concentrations The maximal plasma concentration given in rats by the (toxic) dose of 50 mg/kg daily is 0 236 mg/l (Table V) Concentrations much higher than this have been frequently observed in man A further point of difference between man and rat is the lower persistence of paludrine in the latter, illustrated by the low minimal concentrations, and by the fact that the concentrations determined after only one dose are very similar to those determined after 7 doses

It now became important to determine the blood concentrations associated with chronic toxic effects in other species. We chose to examine the mouse and the chick because the mouse behaved like the rat in the matter of delayed deaths after parenteral injection, whereas the chick did not. Curves for whole blood concentrations in these two species are given in Figs. 2 and 3



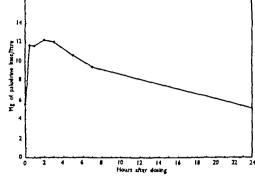


Fig 2—Concentrations of paludrine in the whole blood of mice after the third dose of 30 mg/kg once daily (A) and the fifth dose of 12 5 mg/kg twice daily (B)

Fig 3—Concentrations of paludrine in the whole blood of chicks after the fourth dose of 60 mg/kg twice daily

The curves for mice were determined on one group receiving 12 5 mg/kg twice daily, a treatment which is apparently harmless, and on another receiving 30 mg/kg once daily, a treatment which produces scattered deaths. In the first group measurements were made after the fifth dose, in the second after the third dose. It will be seen that there is a good parallelism between the concentrations toxic for mice and those toxic for rats, and that the general form of the curves is similar, the build-up being negligible. It will also be apparent that the same dose given to mice and rats on a weight basis will produce higher concentrations in the mice.

Curves for chicks were obtained from animals receiving 60 mg/kg twice daily Scattered deaths occur with this regime although about 30 to 50 per cent of the animals will survive indefinitely treatment given for 5 days. Measurements were made after the fourth dose. It will be seen that the peak concentrations associated with a potential lethal effect in chicks are about 10 times as high as concentrations associated with lethal effects in rats and mice. Also the build-up is considerable, a residue of about 5 mg/l being left after the third and fourth doses. The contrast between chicks on the one hand, and rats and mice on the other, is also well shown by the results of other experiments in which only one dose of 50 mg/kg was given to chicks. Peak concentrations of paludrine in the blood rose to 3-4 mg/l and after 24 hours concentrations of 15 mg/l were recorded.

DISCUSSION

Clearly a fundamental difference must exist between the metabolism of paludrine in rats and mice and its metabolism in chicks (and probably man) Certainly, the grosser aspects of distribution which can be measured chemically do not account for the differences in results. In all species examined so far the concentration of the drug in the plasma is about a third to a fifth that in whole blood, and the ratios between tissue and plasma concentrations (which vary from 10 to 100/1 depending on the tissue) are similar. We sought to emphasize the distinction between the chick and the rat, and the fact that the tissue distribution of the drug does not reveal any explanation of it, by comparing the concentrations found in the tissues of rats and chicks following the seventh dose of

50 mg/kg once daily This treatment is lethal for some rats, but tolerated by chicks The blood and tissues of 3 rats or 6 chicks were used at each time interval. The results are shown in Tables V and VI

TABLE $\,V\,$ distribution of paludrine in rats following the seventh oral dose of 50 mg /kg once daily

Time		mg base/l or kg in										
ime	Blood	Plasma	Lung	Spleen	Kidney	Liver	Brain					
Before 1 hour 2 hours 4 " 6 " 24 ",	0 403 0 802 1 07 1 40 0 723 0 320	0 0693 0 0804 0 154 0 236 0 144 0 0671	1 61 4 34 11 2 17 9 6 41 1 82	0 414 2 62 4 69 12 3 1 95 0 711	0 577 3 03 9 14 9 80 2 10 0 947	0 748 20 6 32 4 30 1 11 9 1 07	0 0 trace 0 trace (0 123)					

TABLE VI distribution of paludrine in chicks following the seventh oral dose of 50 mg /kg once daily

Toma		mg base/l or kg in											
Time	Blood	Plasma	Lung	Spleen	Kidney	Liver	Brain						
Before 1 hour 2 hours 4 " 6 " 24 "	3 25 3 78 8 15 7 59 5 91 2 95	1 02 1 31 2 84 2 58 2 14 0 809	72 4 62 9 121 117 103 75 6	25 9 26 6 73 8 57 6 40 9 12 4	92 6 89 7 214 246 177 53 9	45 3 62 8 136 104 95 1 31 6	11 5 10 5 19 4 17 5 18 7 15 1						

Although the treatment is tolerated by chicks, but fatal for some rats, the drug concentrations are uniformly higher in the chicks. It would seem, too, that paludrine reaches the brain more readily in the chick than in the rat, a point which is of interest because, so far as we are aware, such a species difference has not been demonstrated for any other drug. However, this difference would hardly seem to have any bearing on the high blood concentrations in the chick or the delayed deaths in mice and rats. Surveying the results of all the experiments, we have come to the conclusion that the simplest explanation of them is to postulate that paludrine, in mice and rats, is metabolized to a substance persistent in the body and more toxic than the drug itself, in chicks, and probably in man, the metabolism is either different qualitatively or, if it is similar, the degree of degradation to the toxic substance is much less. The relevant facts can be summarized as follows.

1 Equivalent doses of paludrine give higher concentrations, and the drug is more persistent, in chicks than in mice and rats. On the other hand, it is more

toxic for mice and rats than for chicks On the evidence so far available, it is probable that man behaves like the chick rather than like the rat or mouse

- 2 Although paludrine appears to be removed so readily from the blood of mice and rats, delayed deaths may occur in both species and, after parenteral administration, an additive toxic effect can be produced by a second injection given even 24 hours after the first.
- 3 The tissue distribution of the drug in its grosser aspects does not account for the differences in susceptibility between chicks and rats. Concentrations are uniformly higher in the chicks
- 4 The distribution of 4430 (an N_s -methyl derivative of paludrine) in the body is similar to that of paludrine (Spinks, 1946, 1947), but delayed deaths are not associated with its injection into animals
- 5 Recoveries of paludrine from the faeces and urine of rats (and rabbits) are low, usually less than 30 per cent of the dose (Spinks, 1947), which contrasts markedly with what obtains in man, where they are much higher, often up to 60 per cent (Maegraith et al, 1946)

SUMMARY

- 1 Measurements of the toxicity of paludrine for mice, rats, rabbits, and chicks are given
- 2 Delayed deaths follow the intravenous injection of paludrine into mice and rats, and it is noteworthy that an additive toxic effect can be obtained by a second intravenous injection given many hours after the first. Delayed deaths do not follow the intravenous injection of the drug into chicks, and in these animals an additive toxic effect is not produced by a second injection
- 3 Measurements of the concentration of paludrine in the blood of mice, rats, and chicks under various treatments showed that chicks tolerate much higher concentrations of the drug in the body than do mice and rats
- 4 Gross measurements of the drug in the organs of chicks and rats on a similar treatment (50 mg/kg once daily) did not reveal differences sufficient to account for the difference in tolerance. Because of this, and in the light of other evidence which is presented, it is suggested that the metabolism of paludrine in chicks is different from what it is in rats and mice. The facts would be explained if paludrine, in mice and rats, were degraded in large measure to a substance more toxic than the drug itself

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pA. A NEW SCALE FOR THE MEASUREMENT OF DRUG ANTAGONISM

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When the activity of a drue can be expressed in terms of a stable standard which does not differ from it qualitatively, there is no difficulty in getting reproducible results, since all the assays tend to give the same answer, and any degree of accuracy can be attained provided that the experiment is repeated sufficiently often. When, however, the activity of a new drug or drug antagonist has to be defined in terms either of some other drug or of some of its own effects, the results are not equally reproducible since the apparent activity varies in successive experiments, even though conditions are kept as constant as possible. The difficulty of making results of one laboratory available to another is aggravated by the multiplicity of methods used and frequently by the lack of information of their variability; this applies particularly to methods of expressing drug antagonism

It would obviously be of advantage if some common method of expressing drug antagonism could be agreed upon. In the present paper it is proposed to introduce a new measure of drug antagonism, pA, based on a suggestion made originally by Clark and Raventos (1937). Apparatus and methods are described for determining pA accurately on the guinea-pig's ileum, and the activity of several known antagonists of histamine and acetylcholine has been measured in terms of pA. The variability of the pA measure has been estimated and methods are discussed of obtaining reproducible results in the most economical way.

APPARATUS

All the experiments were done on the isolated ileum of the guinea-pig. The apparatus used for assaying drug antagonists is shown in Fig. 1. It consists essentially of a gut both which can communicate with two alternative systems, one of which is filled with ordinary Tyrode solution and the other with Tyrode solution containing the antagonistic drug, the latter solution can be replaced in the course of the experiment without interfering with the assay

All the operations involved in an assay, except the injection of the drug, are performed automatically. The principle of the method has been described before (Schild, 1946). "Telephone relays are converted to compress rubber tubing. When the relays are activated the rubber tubing is decompressed and fluid is allowed to flow. These relays control the emptying and filling and the adjustment of fluid level of an isolated organ bath. They are activated at regular time intervals through a telephone uniselector which makes 12 successive

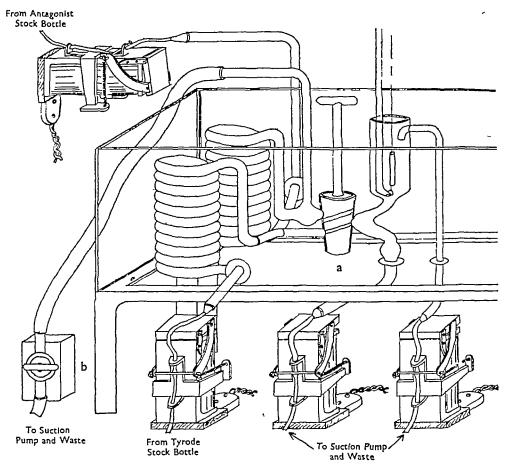


FIG 1—Apparatus for assaying drug antagonists Two stock bottles provide the inflow, one filled with Tyrode solution, and the other with a Tyrode solution of the antagonist drug All the outflows are connected to a water suction pump. The electro magnets compressing rubber tubing are automatically operated through the selector circuit shown in Fig 2. They are standard P.O. telephone relays of 3,000—6,000 ohms resistance operated directly from D.C. mains, with a special armature made in our workshop. The movement of the armature is controlled by two coiled springs which tend to compress rubber tubing of 2×4 mm diameter by means of a thin brass rod.

contacts in a cycle The duration of each contact is usually 15 sec, thus producing a cycle of 3 min. The selector also controls the movements of the drum and a light signal to time the injection of drugs." A diagram of the selector circuit is shown in Fig. 2.*

The present apparatus differs in the following respects from the one previously described

1 An additional inflow relay is provided to control the inflow of the antagonistic solution. The two inflow relays are activated simultaneously, allowing one of the two solutions to flow into the bath according to the position of the 3-way tap. By means of

^{*}All the component parts of the automatic apparatus, including AC mains-operated electro-magnets compressing rubber tubing, and AC-operated 12-step selectors, are now made by Messrs Londex, Ltd

switch D (Fig. 2) the selector can be short circuited and the inflow religs netwated directly If the solution in the system containing the antagonistic drup is to be replaced, the 3-way tap a (Fig. 1) is turned into the null position, switch D is netwited, and tap h is opened, allowing the solution to drain into the exhaust

- 2 Switches A B and C control certain alternative arrangements of the cycle. By means of switches A and B the put both may be emptied and refilled twice in succession instead of only once. By means of switch C the signal may be advanced by 15 sec., permitting a longer period of contact between drug and tissue. Table I shows the intomatic operations performed in a complete cycle together with the possible alternative arrangements.
- 3. The outflows are operated by suction
- 4 A new type of adjustment for telephone relays has been used to compress rubber tubing. The adjustment is somewhat more complicated than the one previously described, but it is more efficient and stable. It is illustrated in Fig. 1.
- 5 Two put biths have been operated simultaneously. The biths were contained in a large thermostatically controlled tank, stirred by a circulating water-pump. As a rule a lag period of 30 sec was maintained between the two cycles, tracings being recorded on two independent drums situated at opposite ends of the tank

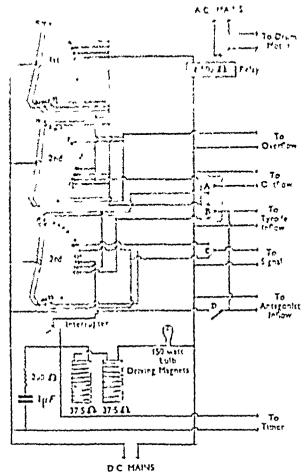


Fig 2—Diagram of selector circuit for one muscle bath

TABLE 1
STEPS IN CYCLE

Relays	1	2	3	4	5	6	7	8	9	10	11	12
Outflow usual alternative	++		+									
Inflow {usual alternative		++		+								
Signal {usual alternative										+	+	
Overflow								+	+			
Drum									+	+	+	+

The writing lever used in these experiments is illustrated in Fig. 3

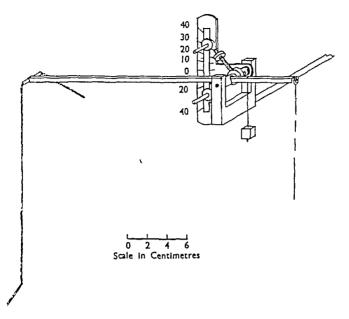


Fig 3 —Approximately linear and isotonic frontal writing lever

It may be asked to what extent these modifications of the usual Burn-Dale isolated organ bath are essential for these experiments

The purpose of the automatic apparatus is to ensure constant time intervals and bath volumes, to enable more than one assay to be performed at the same time and in general to enable the experimenter to divert his attention from servicing the bath and drum. In practice the automatic apparatus has been found very useful, but it is obviously not essential for carrying out these tests since all the operations can be performed by hand

The main purpose of adding the antagonistic drug to the bath fluid before it reaches the gut, instead of adding it in the usual way by injection into the muscle bath, is to prevent the gut coming into contact with pure Tyrode solution during the period of replacement of the bath fluid when the effect of an antagonist on successive contractions is being investigated. The present method is thus particularly suitable for investigating the effect of antagonists in relation to time, whilst both methods are suitable for determining the effect of antagonists on a single contraction. The old method is most suited for preliminary experiments and for comparative measurements.

The purpose of the long writing point used on the frontal writing lever is to ensure that the relation between shortening of the gut and effect on the drum should be linear. This is especially important when the effects are measured in terms of a maximum contraction. The errors obtained with a shorter writing point are, however, usually not excessive, unless a very short writing point is used or the angle of excursion of the lever becomes greater than about 30° from the horizontal, this may be prevented by means of two adjustable stops limiting the excursion of the lever as shown in Fig. 3. The errors may be calculated from the formula given in a previous communication (Schild, 1944). The lever can be made practically isotonic, by means of the screw adjustment shown on the pivot. The adjustment may be tested by suspending an appropriate weight at the point of attachment of the thread. The lever should then balance in every position in which it is likely to be used. The tension exerted by the lever in these experiments was of 600-800 mg.

The following antagonist drugs were used in these experiments

Neoantergan, or N-p-methoxybenzyl-N-dimethylaminoethyl- α -aminopyridine acid maleate (Bovet, Horclois, and Walthert, 1944)

Benadryl, or dimethylaminoethyl benzhydryl ether hydrochloride (Loew, Kaiser, and Moore, 1945)

Pethidine, or ethyl 4-phenyl-1-methylpiperidine-4-carboxylate hydrochloride (Schaumann, 1940)

Atropine sulphate

THE pA SCALE

pA_x is defined as the negative logarithm to base 10 of the molar concentration of an antagonistic drug which will reduce the effect of a multiple dose (x) of an active drug to that of a single dose. Thus, if the presence of a concentration of 10^{-5} molar pethidine in the bath fluid reduces the effect of 2 μ g histamine to that produced, in the absence of pethidine, by 1 μ g histamine, pA₂ pethidine-histamine=58

It is obvious that a constant of this nature can refer only to a given drugantagonist pair acting on a definite pharmacological preparation, e.g., the guineapig's ileum, and that a representative pA value must be the mean of several individual determinations pA values are dependent on the length of contact between antagonistic drug and tissue, but they are apparently independent of the absolute concentrations of the active drug used. These points will be discussed in detail later

Method of determining pA_2 —The principle of the method consists in finding two concentrations of the antagonistic drug such that one will reduce the effect of a double dose of the active drug to slightly less and the other to slightly more than the effect of a single dose. The concentration corresponding to pA_2 is then computed by interpolation on a logarithmic scale

The following results justify to some extent the use of a logarithmic scale for interpolation. In a series of pA₂ determinations a third concentration of antagonist was added to test for linearity between log concentration of antagonist and effect. The points, plotted on a logarithmic scale in Fig. 4, each point representing the mean of several determinations, fall on approximately straight lines.

In order to get reliable results a constant submaximal response to the stimulant drug must be produced before addition of the antagonist, 10–20 preliminary contractions may be required to achieve this At this point the muscle chamber

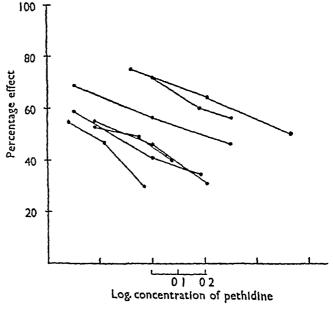


Fig 4—Guinea-pig ileum Pethidine-histamine Approximately linear relation between log concentration of pethidine and reduction of histamine effect

is joined to the system containing a Tyrode solution of the antagonistic drug, which now replaces Tyrode solution when the bath fluid is changed. At the next injection the dose of active drug is doubled. Injections of a double dose of active drug in the presence of the antagonist are continued for several periods (usually five periods) in order to observe whether the effect of the antagonist increases with increasing length of contact. Eventually the bath fluid is switched back to Tyrode solution to test for persistence of antagonistic effect and the assay is concluded by producing a series of maximal effects. A second concentration of antagonist is investigated in the same way, usually on a fresh piece of gut, and the results, expressed in terms of the maximal contraction, are used for interpolating pA2 values after various periods of contact with the antagonist

As in these experiments the automatic apparatus was set to produce intervals of 3 min between injections and pauses of 2 min between completion of change of bath fluid and the next injection, the first pA measurement was made when the antagonistic drug had been in contact with the tissue for 2 min and further determinations at 3 min intervals. During this period the tissue never ceases to be in contact with the antagonistic drug which is contained in the bath fluid itself, and an even flow of stimulation at constant intervals is maintained as the period of contact with the antagonist is gradually increased

Although tedious, the use of a fresh piece of gut for each concentration of the antagonist has been found to be the most satisfactory procedure when the effect of the antagonist is persistent. In our experience, variations in sensitivity to the action of antagonists of different segments of the same gut are remarkably small and are not correlated with variations in sensitivity to the stimulant drug, provided that pieces which are obviously damaged and insensitive are rejected. Although it is advantageous to use two pieces of gut simultaneously, this is not essential, since the guinea-pig ileum does not seem to alter appreciably in sensitivity if left in clear Tyrode solution at room temperature for several hours

Fig 5 (p 205) illustrates two complete pA_2 determinations done on six segments of the same gut. The object was to determine pA_2 values after a short period of 2 min and after a long period of 14 min contact with the antagonist. The following two examples, quoted from the experiment shown in Fig 5, illustrate the method of computing pA_2 .

pA benadryl-histamine (2 min contact)—After 2 min contact between muscle and antagonistic drug a double dose of histamine in 10^{7,94} molar benadryl (1 300 million) produces an effect which is 7 per cent (of the maximal effect) greater and a double dose of histamine in 10^{7,94} molar benadryl (1 100 million) an effect 11 per cent smaller, than that produced by a single dose of histamine in Tyrode By interpolation the molar concentration of benadryl which would just reduce the effect of a double dose of histamine to that of a single dose is 10^{-1,15} (1 195 million) Hence pA₂ (2 min contact)=7.75

 $\int pA_2$ neoantergan-histainine (14 min contact)—When the antagonist has been in contact with the muscle for 14 min (5th injection in presence of antagonist) a double dose of

histamine in $10^{-9.55}$ molar neoantergan (1 9 000 million) produces an effect 7 per cent greater and the same dose in $10^{-9.05}$ molar neoantergan (1 3,000 million) an effect 10 per cent smaller than a single dose of histamine in Tyrode By interpolation pA₂ (14 min contact)=9 36

Independence of pA and concentration of antagonist—Since the absolute concentration of the active drug does not enter into the definition of pA, it was of interest to find out whether the depression produced by a certain concentration of the antagonist was, in fact, independent of the concentration of active drug used. Although this is generally accepted as true (Gaddum, 1937), it seemed worth investigating the point, using a properly randomized experimental design which could be statistically analysed.

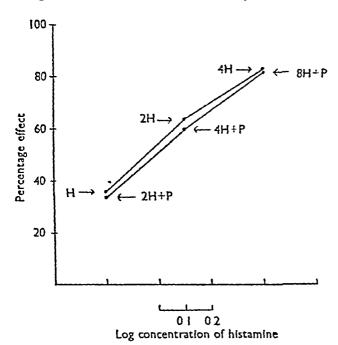


Fig 6—Effect of a constant dose of pethidine (P) on contraction produced by various concentrations of histamine (H) The depressor effect of the antagonist is independent of the contraction produced by the active drug Upper curve histamine alone Lower curve double dose of histamine+pethidine

1

The experiments were carried out as follows Several doses of the active drug alone and of the active drug plus antagonist were administered If the antagonist produced the same amount of depression at each concentration of the active drug, the concentration-action curves in the presence and the absence of the antagonist should be parallel Doses were administered repeatedly in a random order, and the results were analysed by means of analysis of variance for statistically significant deviations from parallelism

Five such experiments were performed, three with pethi-dine-histamine, one with atropine-acetylcholine, and one with pethidine-acetylcholine. One of the experiments is illustrated in Fig. 6, each point on the curve representing the mean of four determinations. It will be seen that at each level the reduction

of the histamine effect by pethidine was approximately the same. There was no statistically significant deviation from parallelism between the two curves. Similar results were obtained in the other experiments

ANTAGONISM OF NEOANTERGAN, BENADRYL, PETHIDINE, AND ATROPINE TOWARDS HISTAMINE AND ACETYLCHOLINE

The activity and relative specificity of these antagonists of histamine and acetylcholine can be conveniently summarized in terms of pA. In Fig. 7 the

two vertical lines represent pA₂ scales for antagonists of histamine and acetylcholine. The activity of an antagonist towards these drugs is indicated by its position on the scale, one scale division corresponding to a tenfold difference in activity. Points on the two scales referring to the same antagonist are joined. If an antagonistic drug does not discriminate between two active drugs, the line joining the scales is horizontal, if it discriminates sharply the line is steep, as with neoantergan and atropine. Neoantergan is the most discriminating of the four antagonists, being 40,000 times as active against histamine as it is against acetylcholine. Atropine is 1,000 times as active against acetylcholine as it is against histamine. Pethidine, on the other hand, discriminates hardly at all between the two

A single pA value is not sufficient to characterize an antagonist fully. To describe fully the relation between a given antagonist and an active drug it would be necessary to state completely both the time-action and the concentration-action relations of the system. Short of this, four characteristic pA values have been selected in the present study which together give some indication of the change of activity of an antagonist with concentration and with time. The

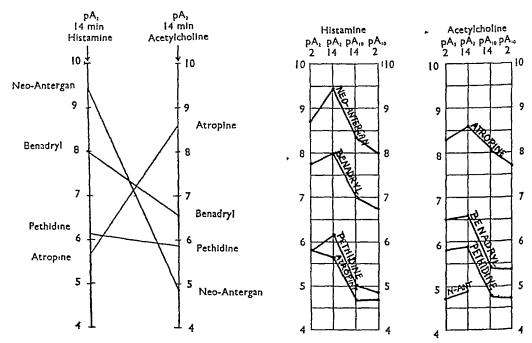


Fig 7—pA₂ scales of histamine and acetylcholine. At the time of measurement the antagonist had been in contact with the tissue for 14 min. The results may be taken to represent approximately equilibrium conditions

FIG 8—Each antagonist of histamine and acetylcholine is characterized by four pA values. Note qualitative differences between antagonists with regard to increase of action with time and pA₂-pA₁, difference

following four pA values have been selected for this purpose the pA₂ values after 2 and 14 min contact and the pA₁₀ values after the same two periods of contact

A 14 min period of contact with the antagonist (corresponding to five consecutive contractions in the presence of an antagonistic drug) has been arbitrarily chosen as representing approximately equilibrium conditions, since at that time there is usually not much further increase of depression. It would, of course, be preferable always to establish true equilibrium conditions, but it was found that in some instances the effect of the antagonist appeared to increase indefinitely, when this happens it becomes difficult to distinguish between the effect of the antagonistic drug and that of a spontaneous deterioration of the preparation, and true equilibrium conditions cannot be established

Atropine—Acetylcholine					Pethidi	nc—Acety lo	choline
	pA ₂	pA ₁₀	pA_2-pA_{10}		pA ₂	pA ₁₀	pA_2-pA_{10}
2' 14'	8 37 8 77	7 72 8 05	0 65 0 72	2' 14'	5 79 5 89	4 69 4 71	1 10 1 18
14'-2'	0 40	0 33		14'-2'	0 10	0 02	

The data are taken from three experiments in which pethidine was tested against acetylcholine and three further experiments in which atropine was the antagonist, each experiment being complete in the sense that all the four pA values were determined in the course of a single assay. When the data are tabulated in this manner the row differences' provide a measure of the steepness of the concentration-action curve, and the column differences indicate changes of activity with time. Pethidine and atropine differ in both these respects. The row differences are of the order of 0.7 for atropine and of 1.1 for pethidine, indicating that in order to compensate for a fivefold rise of acetylcholine the concentration of atropine has to be raised fivefold and that of pethidine thirteenfold. The difference is statistically significant. The differences in the columns show that after 14 min contact the effect of atropine is over twice that after 2 min contact, whilst the effect of pethidine increases hardly at all during this period.

The difference between pA_1 and pA_{10} provides a quantitative test for the hypothesis that antagonists compete with drugs for receptors according to a simple mass action relation. It can be shown that the mass action equation as developed by Gaddum (1937) for a first order reaction requires a ninefold increase of antagonist corresponding to a fivefold increase of active drug between pA_2 and pA_{10} . Straight proportionality between drug and antagonist at low concentrations of the antagonist is presumptive evidence against the existence of a simple mass action relation

The results of pA determinations are summarized in Table II Each drugantagonist pair is characterized by four pA values The figures given are mean values, the total number of individual determinations and their standard deviation being indicated in the Table Fig 8 shows some of the differences between antagonists as revealed by the measurement of four pA values for each from differences in their general activity, antagonists also show characteristic differences in time-action and concentration-action relations The lines joining the outer scales to the two inner scales represent increases of activity with time, these are greatest in the two most active antagonists. The lines joining the two inner scales indicate differences between pA2 and pA10 (at approximately This difference is smallest with the pair atropineequilibrium conditions) acetylcholine, which provides the only instance of straight proportionality between concentration of drug and antagonist In all other cases a tenfold or greater increase of antagonist concentration is required to balance a fivefold increase of drug concentration

One of the most interesting findings has been a complete lack of correlation in the behaviour of the same antagonist when tested against two different drugs. This applies both to time-action and concentration-action relations. Thus, the effect of neoantergan after the first 2 min contact increases little further against acetylcholine, but continues to increase steeply against histamine. As for concentration-action curves, atropine has a steep concentration-action curve against acetylcholine and a relatively flat one against histamine. These results suggest that when the same antagonist antagonizes two different drugs the mechanisms involved may be quite different.

After-effects of antagonists—The rate of recovery after the antagonist has been removed from the bath varies in much the same way as the rate of development of the effects, and here again the same antagonist may be persistent when assayed against histamine and non-persistent against acetylcholine and vice versa. Fig. 5 shows examples of slow (neoantergan) and quick (benadryl) recovery from antagonists on the same preparation

A curious after-effect which occurred at times, especially with neoantergan, is shown in Fig 9 (p 205) the maximum depression of the effect of histamine did not take place in the presence of neoantergan, but shortly after it had been removed from the bath, as if the act of washing out the antagonist had further increased its effect

VARIABILITY OF pA DETERMINATIONS

Determinations of pA₂ pethidine-histamine were made in 19 different experiments spread over a period of over one year. The results were distributed as shown in Fig. 10. The variations of pA indicate a 32-fold (or, omitting one result, 24-fold) variation in the sensitivity of the tissue. The shape of the

distribution curve suggests sampling from a non-homogeneous population. In 10 of these experiments two or more pA_2 determinations were made on the same gut and consequently variation between animals could be compared with variation in successive tests on the same animal by means of Fisher's Z test. The resulting Z value was highly significant, suggesting that there is true variation between guinea-pigs in their sensitivity to antagonists

TABLE II results of pA determinations

The total number of individual determinations is given in parentheses and σ is the standard deviation

Active	Antagonist	p.A	Λ_2	p.A	110
Ad	Antagonist	2 min	14 min	2 min	14 min
	Atropine	$827 (11)$ $\sigma = 011$	8 61 (11) σ = 0 15	$772(3)$ $\sigma = 007$	8 05 (3) σ = 0 13
Acetylcholine	Benadryl	$649(3)$ $\sigma = 007$	6 57 (3) σ = 0 09	$5 36 (2)$ $\sigma = 0 06$	5 4 (2) σ = 0 04
	Pethidine	$579(3)$ $\sigma = 007$	5 84 (4) σ = 0 14	47 (4) σ = 0 05	$476 (5)$ $\sigma = 015$
	Neoantergan	4 71 (2) σ = 0 06	4 86 (2) σ = 0 09		
	Neoantergan	8 71 (4) σ = 0 15	9 46 (5) σ = 0 22	7 99 (1)	8 36 (1)
mine	Benadryl	7 75 (8) σ = 0 1	8 02 (9) σ = 0 28	6 74 (4) σ = 0 11	$702(4)$ $\sigma = 031$
Histamine	Pethidine	5 78 (19) σ = 0 14	6 13 (5) σ = 0 46	$4 84 (5)$ $\sigma = 0 17$	5 0 (5) σ = 0 21
	Atropine	$573(3)$ $\sigma = 022$	$5 64 (3)$ $\sigma = 0.18$	$\begin{array}{c} 4 \ 63 \ (3) \\ \sigma = 0 \ 24 \end{array}$	a = 6 (3) $a = 0.24$

pA measurements are more variable after long periods of contact with the antagonist than after short periods. The following standard deviations summarize the variation encountered for the four different types of pA measurements

	Standard	Degrees
	deviation	of freedom
pA_2 min contact*	0 13	31
pA ₁₀ 2 min contact	0 14	15
pA ₂ 14 min contact	0 25	34
pA ₁₆ 14 min contact	0.22	16

^{*}Omitting pethidine-histamine

The standard deviation is nearly twice as great after 14 min as after 2 min. This is somewhat surprising, since variation might be expected to become less as equilibrium conditions were approached. The increase in variability may be partly due to spontaneous changes in sensitivity of the preparation during the longer period of contact.

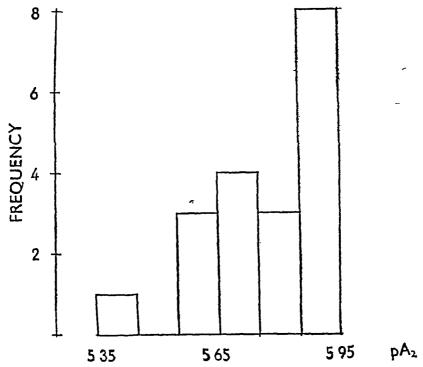


Fig 10—Frequency distribution of pA values for pethidinehistamine obtained after 2 min contact between drug and antagonist, 19 experiments

The Use of Comparative Assays for Determining pA_2

It has been shown that animals vary in their sensitivity towards individual antagonists and that this variation is greater when the period of contact of antagonist with tissue is long than when it is short. It seemed possible that some of this variability might be eliminated in a comparative assay in which one antagonist was pitted against another antagonist. Such an experiment, involving repeated injections of each antagonist, might possibly be carried out on a single piece of gut so long as excessive depression by the antagonists was prevented by keeping the periods of contact short and by giving numerous "recovery" injections of the stimulant drug, after washing out the antagonist.

Six experiments were performed in which the action of pethidine towards histamine was compared with that of atropine on a single preparation. The experimental plan of these assays was similar to one previously used by the author (Schild, 1942) for histamine

assays In the histamine assay only two concentrations of the standard and two of the unknown are used. These concentrations are given in a random order in successive "randomized groups" of four, and the results are eventually computed and analysed statistically on the assumption of a linear relation between log dose and effect. In the present experiments the place of four concentrations of histamine is taken by two concentrations of each antagonist administered 2 min before the injection of a constant dose of histamine. In addition several injections of histamine alone are given following each administration of the antagonist until the sensitivity to histamine has been apparently restored. Fig. 11 shows parts of two such experiments. In both cases a "randomized group" of four doses of antagonist is represented, a complete experiment consisting of several (2-4) such "random-

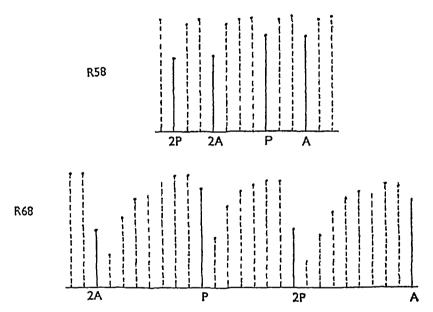


Fig 11—Comparisons of activity of pethidine and atropine towards histamine on a single piece of gut. Extracts from two such experiments. The solid lines represent effects of histamine in the presence of an antagonist which has previously been in contact with the tissue for 2 min, the dotted lines the effects of the same dose of histamine alone administered at 3 min intervals until sensitivity is restored. As far as possible approximately equally depressant doses of pethidine and atropine were used, pethidine being more than twice as active as atropine. The final ratio of activity is calculated by assuming a common slope for the two antagonists and a linear relation between log concentration and effect.

ized groups" In one of these experiments the antagonists produced little after-effect, in the other a prolonged depression necessitating many "recovery" injections. In all the experiments statistical analysis showed satisfactory parallelism between atropine and pethidine slopes. The results of these comparisons, which were done at various times and on different stocks, were remarkably constant. The following figures of the logarithm of the ratio pethidine/atropine were obtained 033, 036, 037, 034, 03, 033

 pA_2 atropine-histamine by direct and inducet method—pA values may be determined directly without reference to another antagonist, or indirectly, by measuring in a comparative assay, such as the one outlined above, the activity

of an unknown antagonist in relation to one whose pA is already established, and computing the unknown pA by adding to the known pA the logarithm of the ratio of molar activity of the two antagonists. Thus

$$pA_2$$
 (atropine-histamine, 2 min)= pA_2 (pethidine-histamine, 2 min) - 0 34 = $5.78 - 0.34 = 5.44$

This method would seem to be economical provided that the results agree with those of direct determinations, this, however, is not entirely the case. There is a slight discrepancy between direct and indirect pA determinations which suggests that, although in comparing the activity of antagonists on a single piece of gut a 2 min period of contact was adhered to, the gut responded in fact if the period of contact had been longer. Indeed, the results agree better with direct pA_2 determinations done after 14 min contact, as the following data show

	pÁ-	Comparison	pA,
	(2 min	on same gut	(14 min
	contact)	(2 min contact)	contact)
Pethidine histamine	5 78		6 13
Atropine-histamine	5 73		5 64
Log ratio pethidine atropine	0 05	0 34	0 49

These results might be explained by assuming that in comparing antagonists on a single preparation they are never completely removed in spite of repeated washings

In comparing two antagonists on the same gut the result may be vitiated by certain systematic errors unless care is taken that the effect of a previous dose has subsided at the time the next dose is added, especially when the two antagonists have different modes of action. The following example illustrates the point

Antagonist (a) is to be compared with antagonist (b), their effects are additive but (a) has a persistent action and (b) a readily reversible action. When (a) is added to the bath some of its effect persists after it has been removed. If a depression is produced by (a) and matched at the next injection by a depression produced by (b) the real effect of the latter, acting as it does on a depressed preparation, is less than it appears. At the next injection the same concentration of (a) reproduces its old action, the effect of (b) having worn off

Comparison of antagonists on separate pieces of the same gut—In these experiments direct pA determinations on two different antagonists were done simultaneously using a separate piece of gut for each antagonist and each concentration, all the segments being taken from the same guinea-pig Variability between guinea-pigs was thus eliminated

An experiment of this kind comparing the action of neoantergan and benadryl towards histamine is illustrated in Fig. 5. The results of this and two further similar experiments are shown in Table III

The following points may be noted

(1) The difference between pA values at 2 min contact is remarkably constant, more constant than the absolute values. This confirms previous conclusions that the gut varies simultaneously in sensitivity towards different antagonists

- (ii) Increase of activity with time (pA. 14 min -pA 2 min) varies, but it is in each case considerably greater with neoantergan than with benadryl
- (111) At 14 min pA₂ values and their differences vary more than at 2 min This confirms previous conclusions

In conclusion it may be said that comparative measurements definitely eliminate a certain amount of variation. Comparisons on a single piece of gut

TABLE III $pA_2 \ \ \text{neoantergan-histamine} \ \ \text{and} \ \ \text{benadryl-histamine} \ \ \text{determined} \ \ \text{in} \ \ \text{the same}$ experiment using a separate piece of illum for each concentration of antagonist

		pA ₂ va	dues benadryl	pA ₂ neoantergan —pA ₂ benadryl
R 205	2' 14'	8 91 9 76	7 94 8 35	0 97 1 41
	14'-2'	0 85	0 41	
R 206	2' 14'	8 61 9 60	7 66 7 78	0 95 1 82
	14'-2'	0 99	0 12	
R.207	2' 14'	8 65 9 36	7 75 8 26	0,90 1 10
	14′-2′	0 71	0 51	

are efficient in the sense of allowing many determinations to be made in a relatively short time, but they may be affected by systematic errors owing to persistence of antagonistic effects. Comparisons on separate pieces of gut are laborious but free from objections and give a more complete picture, since the time factor may be taken into account.

DISCUSSION

Clark and Raventos (1937) proposed to use as a measure of activity of an antagonist the concentration which would neutralize the effect of a tenfold increase of active drug. One of the chief merits of this measure is that, being a null measure, and involving no change in response, it is independent of the method of experimentation used, and yields results on different preparations which are directly comparable

pA is based on the same idea as the measure adopted by Clark and Raventos, but it is expressed in a rather more convenient form. It may be used to define the activity and specificity of an antagonist, its time-action relations and the trend of its concentration-action curve. pA values are additive, for instance, in order to express the total activity of a compound against both histamine and acetylcholine, the respective pA values may be added together.

The activity of an antagonistic drug may be expressed in one of two ways, by reference to another antagonist or by some measurement not involving a comparison with another drug. Since the activity of a drug cannot as a rule be expressed accurately in terms of another drug which is qualitatively different, the comparative method gives an incomplete and often misleading picture of the activity of an antagonist. Moreover, unless a definite common standard has been agreed upon, results from different laboratories are difficult to correlate Comparisons may nevertheless be useful in practice owing to their greater economy of time and effort, especially when a series of related compounds is being investigated within the same laboratory. This question has been discussed in detail above

The real test of the usefulness of a measure of drug antagonism is whether the results can be reproduced in another laboratory Variations occurring within the same laboratory can be overcome by repeating the experiment and by random selection of experimental animals, but systematic variations between different laboratories present a more difficult problem At present it is not known whether pA will be affected by such systematic variations or whether results in different laboratories will fall within the range of variations obtained with a highly mixed stock of animals in an individual laboratory If important variations in sensitivity between laboratories should occur, one way of eliminating them would be to use in this type of work a homogeneous strain of guinea-pigs, available to all the laboratories concerned Failing this, it might become neces sary to fall back on the method of establishing differences of pA between antagonists rather than absolute pA values in the hope that the former would be less subject to laboratory variation than the latter The use of an agreed measure of drug antagonism should, at least, make it easier to detect the occurrence and extent of such variations

SUMMARY

- 1 Clark and Raventos (1937) suggested a method of estimating antagonistic power in terms of "the concentration of antagonist which altered by a selected proportion (e.g., tenfold) the concentration of an active drug needed to produce a selected effect" The negative logarithm of this (molar) concentration has been termed pA_{τ} , where τ is the proportion selected. On the guinea-pig's ileum the value of pA appears to be independent of the degree of contraction produced by the active drug. Methods are described for determining the value of pA
- 2 pA is a statistical constant. To obtain a representative value of pA for a given tissue, antagonist, and active drug, the mean of a random sample of determinations on different individual animals must be determined. pA values were found to vary by 0.4 to 0.5 of a unit when the period of contact of the muscle with the antagonist was short, at long periods of contact variability was increased. Variability may be reduced by comparing the activity of one

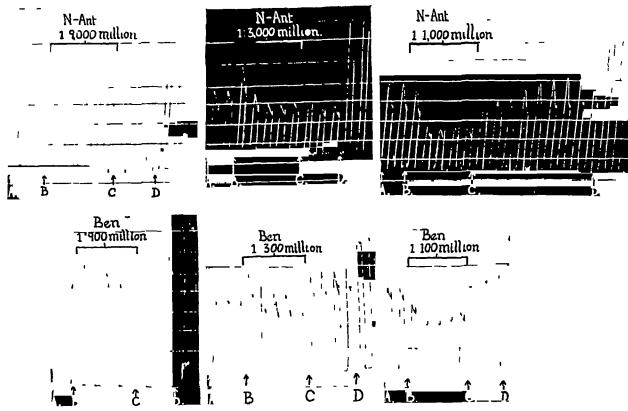
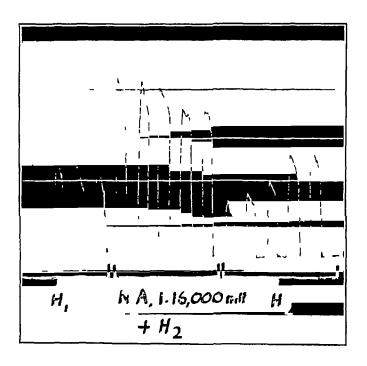


Fig 5—Neoantergan-histamine and benadryl-histamine Complete pA assay The whole assay of the two antagonists was done on the same animal, a separate piece of ileum was used for each concentration of antagonist Before the addition of the antagonist the preparations were stabilized by a series of preliminary histamine injections A-B 0.5 μ g histamine, Tyrode B-C 1.0 μ g histamine, antagonistic solution C-D 1.0 μ g histamine, Tyrode D maximum effects Bath volume=18 c c

Fig 9—Neoantergan-histamine After-effect of antagonist Immediately after removal of the antagonistic drug from the bath there is a further increase of depression H_2 =double dose of histamine



antagonist with that of another Methods are described for making such comparisons in an efficient way and possible objections to a comparative method are discussed

3 The activity of neoantergan, benadryl, pethidine, and atropine in antagonizing histamine and acetylcholine has been determined in terms of pA on the guinea-pig's ileum. All these antagonists act against both histamine and acetylcholine, though at widely differing concentrations and in a qualitatively different way, as shown, for instance, by the time taken for equilibrium conditions to be reached. By characterizing each drug-antagonist pair by four pA values a more complete picture can be obtained of activity as influenced by duration of action and by concentration

I am indebted to Dr F Bergel, of Roche Products Ltd for supplying Pethidine, to D J S White, of Parke Davis & Co for Benadryl, and to Dr R Wien, of May & Baker, for Neoantergan. The special armatures for converting PO relays into fluid switches were designed and made by Mr B F Ballhatchet. The drawings are by Mr A Boura

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SOME ACTIONS OF β-HYDROXY-2β-DIPHENYLETHYLAMINE

BY

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Morphine being a pharmacologically active derivative of phenanthrene Dodds, Lawson, and Williams (1944a) sought a morphine analogue on the same lines as their discovery of stilboestrol—an intensely active analogue of another phenanthrene derivative, oestradiol They found that diphenylethylamine and seventeen related compounds possessed in differing degrees some of the properties of morphine, these were depression of righting reflex in rats, elevation of blood sugar in rabbits, and hyperexcitability, pupil dilatation, and vomiting in cats Some compounds of this series also possessed some analgesic properties when Of these the most promising was diphenylethanolamine or tested clinically β -hydroxy- $\alpha\beta$ -diphenylethylamine, which may be considered to have the same structural relation to morphine as stilboestrol has to oestradiol Later (Dodds et al, 1944b) it was shown to be effective only in cases where the pain was associated with pressure on nerves The possible therapeutic uses of this substance, called M4 in their series, justified an investigation of its general pharmacological actions

Tiffeneau, Levy, and Boyer (1928) found that diphenylethanolamine caused weakening of the beat and slowing of the isolated snail and frog heart, and also of the exposed heart of chloralosed dogs. The substance also caused relaxation of isolated intestine, transient fall of arterial pressure in chloralosed dogs, but gave a vasoconstriction of the perfused isolated frog leg. Hasama (1930) showed that the fall of blood pressure induced by the substance in urethanized rabbits was still obtained after vagotomy or atropine. The vessels of the isolated rabbit ear were dilated by diphenylethanolamine, even in the presence of atropine, since the dilator action could antagonize barium chloride vasoconstriction. Hasama suggested that the activities of the substance were the result of a direct toxic action on smooth muscle. Tainter (1933) and Dodds,

Lawson, and Williams (1944a) also noted the depressor action and the latter authors commented on the dilatation of the pupil and general hyperexcitability of unanaesthetized cats following intramuscular injection of the substance

In this paper is presented confirmation and extension of the previous work with an analysis of the site of action of the substance. The actions of β -hydroxy- $\alpha\beta$ -diphenylethylamine hydrochloride, which will be referred to as M4, were tested on the following mammalian tissues: (a) small intestine, (b) cardiovascular system, (c) the pupil of the eye

METHODS

For studies on isolated intestines and heart, rabbits were killed by stunning Pieces of small intestine were suspended in a bath of oxygenated Tyrode solution (formula in Bayliss, 1924) at 37–38° C and pH 74, and the contractions of the longitudinal muscles recorded The coronary arteries of the heart were perfused with oxygenated Ringer-Locke solution (formula in Bain 1938) at 37–38° C and pH 74, through a cannula in the aorta, movements of the right ventricle were recorded, the heart being steadied by pinning the apex For perfusion of the hind quarters, rabbits and cats were used, the former were killed by stunning, the latter had been anaesthetized with chloralose (60–80° mg/kg intravenously) for other experiments. A cannula was inserted in the lower aorta and the legs were perfused with Ringer-Locke solution at 38° C and pH 74 by a Dale-Schuster perfusion pump, the inflow pressure being recorded with a mercury manometer.

Blood pressure changes were recorded in cats anaesthetized with chloralose (60 mg/kg intravenously in preliminary ether anaesthesia) or with nembutal (0.5 c.c. nembutal solution (Abbott)/kg intraperitoneally). Carotid arterial pressure was recorded by a mercury manometer. To assess the action of M4 on the vessels of different tissues the volumes of a hind paw, of the opposite skinned hind leg, and of a 2-in length of small intestine were recorded optically on photosensitive paper (Downman, Goggio, McSwiney, and Young, 1943) in these experiments the arterial pressure was also recorded optically on the same paper. The organs were enclosed in plethysmographs, the leg plethysmograph enclosing the skinned leg from upper thigh to ankle, but the skin of the same paw and its venous drainage were left intact outside the plethysmograph

For experiments on the pupils of cats, slit-like pupils were produced in two ways First the animals were anaesthetized with chloralose (80 mg/kg intravenously) given in preliminary ether anaesthesia (McDowall, 1925, Bain, Irving and McSwiney, 1935) Secondly, under ether anaesthesia cats were decerebrated through a trephine opening in the cranium, the plane of section curving downwards and forwards from the upper edge of the inferior colliculi to the sphenoid eminence, leaving the hypothalamus and adjoining structures intact. The carotid arteries were temporarily occluded by clips and the vertebral arteries by digital pressure, but they were released as soon as possible. As the animal excreted its ether the pupils closed down to slits. If restoration of blood flow to the brain stem was delayed too long the pupils might not constrict. The occulomotor nerve could be exposed by tearing the dural covering over it on its way to the orbit.

Stock solutions of M4 were made by dissolving the hydrochloride in distilled water, without heat to give 2 or 5 per cent (w/v) solutions. Dilutions were made from the stock solution into the appropriate physiological salt solution. It should be noted that solutions of M4 are acid, and simple neutralization throws the base out of solution. All doses are in terms of the hydrochloride. Injections into the cat were made into the superficial vein of the right foreleg.

(a) Small intestine

RESULTS

Isolated small intestine of rabbit was relaxed by M4 and the rhythmic movements diminished in amplitude. The lowest concentration found effective was 1 in 20,000. The loss of tone was rapid at first, but was followed by prompt recovery of tone and activity on washing (Fig. 1). M4 was active on the

atropinized intestine and antagonized the spasm produced by acetylcholine, eserine, prostigmine, and barium chloride. Whereas M4 relaxed the gut, similar concentrations of morphine sulphate produced a slow increase of tonus

Aqueous solutions of M4 being acid—eg, pH 53 for 2 per cent (w/v) solution—it might be argued that the spasmolytic action represents no more than the action of an acid solution there was a noticeable fall of pH, shown by adding phenol red to the Tyrode solution, after addition of an active quantity of M4 Advantage was taken of the buffering power of plasma The pH of heparinized rabbit plasma was 740 After the addition of 1 volume of 2 per cent (w/v) aqueous solution of M4 to 4 volumes of plasma the pH of the mixture, measured with a glass electrode pH meter, was the same as that of the original plasma The buffered M4 produced the same changes of intestinal activity as unbuffered M4, when each was added to the Tyrode bathing the intestine to the same final concentration of M4

(b) Cardiovascular system

Isolated rabbit heart —01 to 02 c c of 2 per cent (w/v) solution of M4 injected quickly into the perfusion cannula produced a sharp decrease of amplitude, with not more than 15 per cent slowing of the beat. The heart recovered steadily in the

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Fig 1—Action of hydroxydiphenylethylamine
upon longitudinal movements of rabbit duodenum suspended in
Tyrode solution M4=
addition of 2 per cent
solution to give a final
solution of 1 5,000 W=
wash Time signal =
30 sec

next 5 minutes Similar results were recorded when the same doses of M4 were diluted with 0 8 c c rabbit plasma before injection, whereas plasma alone usually produced a slight increase in the amplitude of beat

Faradizing a vagus nerve supplying the isolated heart caused slowing and weakening of the beat. At the height of the M4 action vagal stimulation produced no or but slight slowing of the beat. It was noticed also that the amplitude of the beat might be a little increased during the stimulation

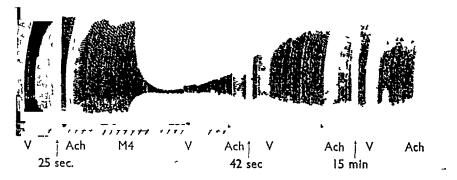


Fig 2—Action of hydroxy-diphenylethylamine on the isolated perfused rabbit heart. Tracing shows movements of right ventricle. M4=injection of 2 mg hydroxy-diphenylethylamine HCl into aortic perfusion cannula, buffered by mixing 1 vol 2 per cent solution of M4 with 4 vol rabbit plasma. Ach=injection of 2 \mu g acetylcholine in 0 2 cc. Ringer-Locke solution. V=faradic stimulation of vagus nerve on the oesophagus. Time tracing=5 sec.

(Fig 2) The vagal inhibitory action returned as the heart itself recovered from the influence of the M4, but the full return of vagal response was not seen until some 5 minutes after the amplitude of the heart beat had fully recovered. The response to acetylcholine—e g , 1 μ g in 0 1 c c. Ringer Locke—was also reduced, and vagal and acetylcholine action was recovered at about the same speed M4 action on the heart was not influenced by prior atropinization. Following

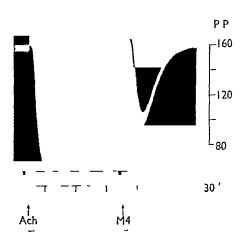


Fig 3—Vasodilator action of hydroxydiphenylethylamine and of acetylcholine compared in cat hindquarters perfused Ringer-Locke solution, pH 75, containing 1 500,000 adrenaline Ach=3 μg acetylcholine M4=2 mg hydroxy-diphenylethylamine HCl Time signal=30 sec Perfusion pressure (PP) in mm Hg

a depressant dose of M4 the action of adrenaline was reduced but not abolished

Perfused hind limb -In order to demonstrate the action of M4 or any vasodilator drug the tone of the vessels was raised by adding adrenaline to the perfusion fluid in a concentration of 2×10^{6} to 3×10^{7} M4 now produced a transient dilatation of the limb vessels, shown by a fall of the perfusion pressure head (Fig. 3) dilator response, which was mimicked by 0.05 to 0.1 cc N/10 hydrochloric acid, cannot be attributed solely to the injection of an acid solution obtained when the M4 was buffered adequately with plasma, as described above, although plasma injected alone produced a small rise of perfusion pressure

The dilator action of M4 was seen when the limb vessels were constricted not only by adrenaline but also by posterior pituitary extract or barium chloride

Vascular responses in anaesthetized cats—Intravenous injection of M4 dissolved in 0.9 per cent sodium chloride solution produced a temporary fall of general arterial pressure (Fig 4) With small doses arterial pressure recovered

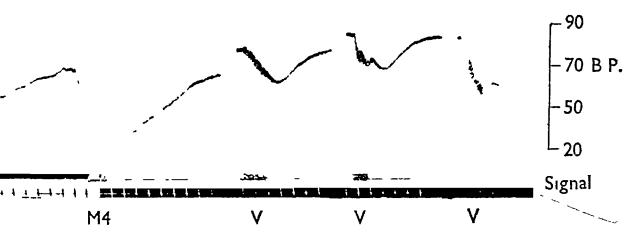


Fig 4—Depressor action of hydroxy-diphenylethylamine, 20 mg iv, upon carotid arterial pressure of chloralosed cat (Signal for injection is 10 sec late) V=faradic sumulation of peripheral end of cut right vagus nerve in neck. Time tracing=10 sec BP calibration in mm Hg

quickly and was usually followed by a small but prolonged hypertension. In chloralosed cats 10 mg M4/kg produced about a 40-mm Hg fall of blood pressure with recovery in two minutes, while 50 mg/kg led to a 75-mm Hg fall of pressure with cardiac irregularity. Doses over 50 mg/kg caused cessation of breathing for periods up to 25 minutes

Accompanying the fall of arterial pressure there was a rapid decrease of volume of the paw, skinned limb, and intestine. At the same time the pulsations in these organs decreased in amplitude. As the arterial pressure recovered the organ volume and the amplitude of the pulsations returned. Similar changes were seen after bilateral vagotomy in the neck and inactivation of both carotid sinuses by tying the arterial trunks close to the sinus where they entered and left the structure. The shrinkage of the peripheral organs started at the same time in each of them and did not commence until the central arterial pressure had already fallen a little (Fig. 5)

The loss of vagus action in the presence of M4 could be shown in the intact animal by comparing the depressor response to faradizing the peripheral end of the cut right vagus nerve in the neck before and after injection of a dose of M4 which produced a prolonged action. The fall of pressure became smaller and the vagal inhibition of heart rate was much reduced, as in the isolated

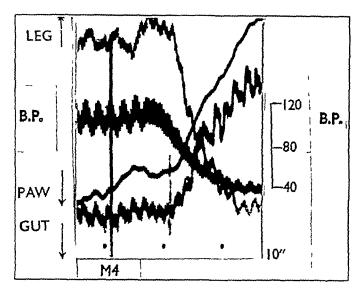


Fig 5—Optical record of carotid arterial pressure, with volumes of left hind paw, skinned right hind leg, and segment of lejunum Cat nembutal ip At signal 30 mg hydroxy-diphenylethylamine iv Time marking=10 sec BP calibration in mm Hg Arrows show direction of vasodilatation

heart, as blood pressure recovered so the vagus responses recovered, but the latter was not complete until some 1 to 5 minutes after the end of the M4 depressor response (Fig 4)

(c) Action on the pupil

In the chloralosed or decerebrated cat with slit-like pupils intravenous injection of M4, in doses of 12 mg/kg or more, produced a rapid wide dilatation of the pupil with slow recovery The whole effect lasted 10 to 30 minutes, and was not altered by cutting both cervical sympathetic chains in the neck In cats with one oculomotor nerve severed it was possible to follow the dilator action of M4 upon the contralateral normal eye, and at the same time to test the ability of the oculomotor nerve to constrict the pupil by faradizing the peripheral end of the cut nerve With the nerve severed the pupil was widely dilated, but constricted to a slit when the nerve was stimulated the height of the M4 action, as judged by the dilatation produced in the normally innervated eye, the constrictor effect of oculomotor stimulation was quite absent As the pupil dilator action of M4 receded, so the ability of oculomotor nerve impulses to produce pupil constriction returned Even when oculemotor action on the pupil was paralysed, stimulating the nerve still produced the usual rotation of the eyeball and enophthalmos Stimulating the peripheral cut end of the cervical sympathetic chain in the neck produced a further slight dilatation

of the pupil when the latter was apparently fully dilated by M4, as well as movement of the nictitating membrane

M4 could antagonize the action of eserine. This was shown by cutting the oculomotor nerve on one side and then constricting the paralysed pupil by instilling into the conjunctival sac 0.65 mg eserine sulphate (1 Burroughs Wellcome "Tabloid"). Intravenous injection of a dose of M4, which produced full dilatation of the normal pupil, produced one-third dilatation of the eserinized pupil.

Stereoisomers of hydroxy-diphenylethylamine

There being two assymmetric carbon atoms in the molecule two optically inactive stereoisomers are possible, the normal form (M4 itself) and an iso form. Each of these can be resolved into two optically active enantiomorphs. A comparison of the activities of these various isomers was attempted, using rabbit jejunum as the test object. More regular responses were produced if the gut was brought into high tone by suspending it in Tyrode solution to which eserine sulphate was added in a concentration of 1.4 million. The isomers were added to the gut bath as 2 per cent (w/v) solutions in water in an amount producing about half the maximum relaxation of the gut. The gut was exposed to each isomer for 5 minutes, then washed twice with Tyrode solution in the next 10 minutes. Even with this long exposure some pieces of gut did not reach their final length as the relaxation was rapid for the first two or three minutes and then proceeded very slowly

Comparing the relaxation produced by similar concentrations of the isomers, 1 15,000, the activities of the isomers could be listed as l-iso>d-normal>d-iso >dl-normal>d-iso. The l-normal form was not available. Equal relaxation was produced by 1 20,000 of the l-iso and 1 12,000 of the d-iso. It is difficult to assess the value of these results. Although there is a consistent difference between the l-iso and d-iso form, the activities of the first four isomers listed are very similar. Comparison of the vasodepressor action in the chloralosed or spinal cat was less successful. The responses to similar doses of the isomers were not always consistent, and might change during the experiment, but the results did not suggest any such difference of activity as shown by the gut

Discussion

Although hydroxy-diphenylethylamine (M4) apparently resembles morphine in having analgesic action, Dodds, Lawson, and Williams (1944) showed that these two substances probably act in different ways. Similarly, the previous results show that the general activities are also different. M4 is in general a depressor of smooth muscle action. Also it has an action which may for the moment be called "atropine-like"

It has been shown that the drug relaxes intestinal muscle and reduces the amplitude of the rhythmic contractions. This action is reversible and can be exerted against substances, such as barium chloride, which raise the tonus of the muscle. A similar spasmolytic action is produced in the blood vessels. These actions might all be explained by a general toxic action which directly reduces the power of the muscle to contract. Such a depressant action is seen in the heart, where M4 reduces considerably the amplitude but not the frequency of the beat. That this effect is not due to a parasympathomimetic action of the drug is shown by its occurrence in the atropinized heart. These findings agree with the reports of previous workers.

In view of the depressor action of M4 it could be argued that the fall of arterial pressure is a consequence of a peripheral vasodilatation, especially as M4 does have such an action in the isolated perfused limb The simultaneous recording of paw, skinned limb, and intestine volumes shows however that in the whole animal the fall of arterial pressure is accompanied by a shrinkage of Since M4 is an active vasodilator in the isolated perfused limb, with maintained inflow, it seems that in the whole animal there is produced a passive vasoconstriction consequent upon the fall of arterial pressure confirmed by the fall of pressure starting demonstrably earlier than the volume changes of skin, skeletal muscle, or intestine The initial rapid fall of blood pressure on injecting M4 seems, therefore, to be due to a reduction of heart output because of the toxic action of the drug on the heart muscle This toxic action is well shown on the isolated heart preparation

The dilator action on the pupil in the unanaesthetized cat might be due to some central action in the brain stem or to a peripheral paralysis of the iris That the neuromuscular mechanism of the iris is influenced directly is shown by the ineffectiveness of the oculomotor nerve stimulation in the presence of a dilator concentration of M4 It is probable, therefore, that a peripheral action would explain the pupil dilatation observed by Dodds and his colleagues is to be noted in the paper by Dodds, Lawson, and Williams that a dose of M4 which produced general hyperexcitability (20 mg/kg intramuscularly) was less than the pupil-dilating dose (50 mg/kg) A general toxic action on the whole neuromuscular mechanism does not seem to be an adequate explanation of the oculomotor paralysis The dilator action of the sympathetic fibres was still present, arguing against an mability of the muscle fibres of the iris to react to nerve impulses, further, conduction in the oculomotor nerve was not impaired because it could still carry nerve impulses to the extraocular muscles, producing movement of the eyeball The action of M4 on the pupil seems to be of an atropine-like nature, blocking conduction at the parasympathetic terminals This action is, however, of short duration and is produced only in concentrations which have direct toxic actions on other smooth muscle

The ability of M4 to block the action of parasympathetic nerve impulses

is seen also in its effect upon the vagal control of heart rate. This action has been observed only after doses of M4 sufficient to produce severe weakening of the beat, but does persist for a few minutes after the visible signs of this depression have worn off It will be recalled that certain barbiturates produce a similar transient blocking of parasympathetic impulses and there is some evidence that the site of the block may be either in the ganglion or at the Thus Koppanyi, Linegar, and Dille (1935) showed neuromuscular junction that some barbiturates, other than thiobarbiturates, produced a transient loss of vagal action and the evidence suggests that the site of action of the drug is mainly at the vagus ganglion, Stravraky (1931) showed that amytal paralyses the submaxillary glands to chorda tympani stimulation, and since the acetylcholine response is also paralysed it appears that the drug acts on the nerve terminals or the secretory cells Garry (1930) also has shown that amytal temporarily abolishes the action of the vagus nerve on the heart in cats and rabbits, the effect passing off much more rapidly in the rabbits, the action of acetylcholine on the frog heart was unchanged, but vagal activity was not tested at the same time in this animal. In the heart both vagal and acetylcholine activities are paralysed by M4 to the same degree, but whether this should be compared with the action of atropine is debatable, since M4 is so toxic to the heart muscle is possible that the effect represents no more than a general toxic action which takes a little more time to recede from the more fragile place where acetylcholine The observations on the pupil, however, suggest that toxic and "atropinelike" action may be separate entities It is curious that although M4 reduces the force of the heart beat very greatly it has relatively little action on the rate of firing of the pacemaker

Although many of the actions of M4 may be due to non-specific depression of the tissues, there seems to be stimulation of some parts of the central nervous system. General bodily activity is increased (Dodds et alia, 1944), an action shown by diphenylethylamine (Tainter, Ludvena, Lackey, and Neuru, 1942), some related compounds will even cause convulsions in cats. Some of the diphenylethylamine compounds also cause a rise of blood sugar (Dodds et alia, 1944), but the ability to cause hyperexcitability and raise the blood sugar are dissociated in M4, this substance producing hyperexcitability without hyperglycaemia. Clearly it would be of interest to know more of the cause of the hyperglycaemia provoked by other diphenylethylamine derivatives.

SUMMARY

A morphine analogue, β -hydroxy- $\alpha\beta$ -diphenylethylamine, has the following actions

1 It relaxes isolated intestine, even in the presence of acetylcholine, eserine, prostigmine, or barium chloride

- 2 The contraction of the isolated heart is reduced in amplitude, this action being unaffected by previous atropinization. Loss of response to vagal impulses and to acetylcholine is also produced
- 3 It causes a fall of arterial pressure, with peripheral vasoconstriction. The latter is a passive effect. In the isolated perfused limb active dilatation is produced.
- 4 It produces pupil dilatation in chloralosed and decerebrated cats by an action on the oculomotor nerve terminals in the iris
- 5 These results might be explained by a direct toxic action of the drug on the active tissue

This investigation was undertaken after a suggestion by Prof E C Dodds, MVO, FRS, whom I have to thank for original supplies of M4 and its isomers. The M4 was made by Boots Pure Drug Co, Ltd, to whom thanks are due. During the work much valuable assistance was given by Miss J G Emmett, of Boots Pure Drug Co, Ltd, working in this laboratory

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THE THERAPEUTIC ACTION OF SOME KNOWN AMOEBICIDES IN RATS

BY

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(Received March 31 1947)

Three main types of drug are used in the treatment of human amoebiasis firstly, drugs of the emetine type, e.g. emetine hydrochloride, emetine bismuth iodide, and auremetine, secondly, arsenical drugs, e.g. carbarsone and stovarsol, and thirdly, halogenated hydroxyquinolines, e.g. chiniofon, vioform, and diodoquin. Opinions differ concerning the respective merits of these three types, indeed the general opinion seems to be that no one type is entirely satisfactory in itself, and that the best clinical results are obtained by the judicious use of all three. As we now have a technique for evaluating the anti-amoebic properties of drugs, using experimentally infected rats (Jones, 1946), it was considered of interest to make a close comparison of the most commonly used of the above types. Such a comparison would also serve to supply standards against which any newly discovered anti-amoebic drug could be compared.

The drugs selected for the comparison were emetine hydrochloride, chimofon, stovarsol, carbarsone, and diodoquin. They were compared when given as a single dose, and also when given according to a multiple-dose schedule

EXPERIMENTAL WORK

Several experiments were carried out, each involving the use of 144 recently weaned rats weighing approximately 20–33 g. In each experiment the rats were separated into six groups of matched weights. They were then injected intracaecally, after laparotomy, with 0.25 c.c. of a suspension of *Entamoeba histolytica* in 5 per cent gastric mucin. The amoebae were cultivated in the enriched serum-buffered saline medium described previously (Jones, 1946).

Two different dosage schedules were employed in the first a single dose was given 24 hours after the operation, and in the second doses were given 24, 30, 48, 54, and 72 hours after the operation. All doses were given orally, by means of a metal catheter. Emetine and chiniofon were given as solutions in water, and stovarsol, carbarsone, and diodoquin as finely dispersed suspensions made by ball-milling for several hours with a suitable dispersing agent (1 per cent Dispersol OG, ICI)

In order to assess the therapeutic effect of the test drug the rats were killed six days after the operation. After this time the infection in the control group is maximal, and any therapeutic effect in treated groups is therefore most readily detectable. Careful postmortem examinations were made of each rat in the experiment, and according to the degree of infection found a score was allocated. The six standard degrees of infection are shown in Fig. 1

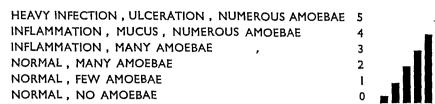


Fig 1 -The degree of amoebic infection found in the caecum of experimentally infected rats

An average degree of infection (ADI) was calculated for each group by finding the average of the individual scores

The statistical significance of a treatment effect may be assessed according to the following formula

$$z = \frac{x - y}{\sqrt{\frac{\sigma^2 x}{m} + \frac{\sigma^2 y}{n}}}$$

where x and y are the ADIs for the control and treated groups respectively

 σx and σy are the standard deviations for x and y respectively, and m and n are the numbers of rats in the control and treated group respectively

The values of σx and σy used in the above formula were read from a curve derived from the results of a large number of control and treated groups (Jones, 1946)

In this series of experiments most of the drugs, when given at the highest dose, showed a therapeutic effect of high statistical significance (P<0.01). This value (P<0.01) was therefore taken as the standard representing definite positive therapeutic effect (+). Therapeutic effects of significance P=0.05 to P=0.01 were regarded as indicating slight though definite therapeutic effect (±), whilst effects of P>0.05 were regarded as indicating insignificant therapeutic effect (-)

The results of the comparisons are recorded in detail in Figs 2 and 3, and summarized in the Table

	CONTROL	EMETINE	CHINIOFON	STOVARSOL	CARBARSONE	DIODOQUIN
-13	NO THEATMENT	20 mgm/Kg	1000 mgm/kg	1.000 mgm/kg.	1000 mgm/kg.	1000 mgm/rg
1 2	ADL 26	VQ1 Q-8	AD-1 0 3	A.D10-6	ADIOB	A0117
	NO TREATMENT	to mgm/Kg.	500 mgm/Kg.	\$00 mgm/Kg.	500 mgm kg.	500 mgm Mg
- 1	ADI.21	ADIO6	ADIO2	ADLI 4	81,104	ADJ 20
NOL						
딾	NO TREATMENT	5 mgm/Kg.	250 mgm. Kg	250 mgm/Kg.	250 mgm/kg	250 mgm/kg
불	ADI 20	A.DJ O 6	ADJ 0-B	ADIL6	A.01.19	AD1,19
8						
DECA	NO TREATMENT	2 5 mgm/Kg.	125 mgm/kg	DOSAGE - 24 HRS. A	FTER INFECTION ORAL	
	A.D1,4-0	ADI 0.7	A0127	A.D.L - AVERAGE	DEGREE OF INFECTION	ų.
	NO TREATMENT	2 mgm/Kg	100 mgm.kg.			
	1 70000	AD1.0-8	ADJ 17			
-13						

Fig 2—The effect of drugs on experimental amoebiasis in rats Single-dose therapy

- '	- CONTROL	EMETINE	CHINIOFON	5TOVARSOL	CARBARSONE	חוטסססטוט
1 4	NO TREATMENT	5×5 mgm/Kg.	5×1,000mgm/kg	5×1000 mgm/kg	5x1000 mgm/Kg	5x1,000 mgm/kg.
3 2	AD122	A.D1 0 2	A0103	A0106	AD104	A.D.J. 0.4
1 8		աստասար				
- NO	NO TREATMENT	5x25mgmyKg	5x500 mgm/Kg	5 x 500 mg mu Kg.	5×500mgmJKg	5x500 mgm/Kg.
ECT.	ADI 27	A.01 0 S	A.D.I 0.9	ADI13	A.D1 07	ADIOS
INF	,					
30 3 30 3	NO TREATMENT	5x125mgm/Kg.	5 x 250 mg m/Kg	5x250mgm/Kg	5 x 250 mgm/Kg	5x250mgm/kg
CRE	AD1 2 0	AD107	A.0.1 2	A.0.1 2.5	A.D.1 2 2	AD128
90						
4	NO TREATMENT	5x0 62 mgm/Kg	5 x 125 mgm/Kg.	DOSAGE ~ 24 30 48	54 % 72 HRS AFTER	INFECTION ORALLY
2	A0115	AD106	AQJ18		SE DEGREE OF INFE	' ' [
	`L					

Fig 3—The effect of drugs on experimental amoebiasis in rats Multiple-dose therapy

TABLE

THE EFFECT OF DRUGS ON EXPERIMENTAL AMOEBIASIS IN RATS

		/kg orally r operation	Significance of treatment $+ = P < 0.01 + P > 0.05$					
•	Emetine	Chiniofon, etc	Emetine	Chiniofon	Stovarsol	Carbarsone	Diodoquin	
Single dose therapy	20 10 5 2 5 2 0	1000 500 250 125 100	+ + + + -	++++-	+ -	+	± 	
	orally 24, 30 72 hr afte	, 48, 54 and r operation						
Multiple dose therapy	5 2 5 1 25 0 62	1000 500 250 125	+ + ±	+ +	+ ± -	+ + 	+ +	

DISCUSSION

In comparing the results of our experiments in rats with the results obtained in human amoebiasis with the same drugs, we must consider certain important differences between the infections in the two species. Whereas in rats the infection is an acute one and is reasonably standardized, in human amoebiasis there is not only the acute disease but also a chronic phase of widely varying symptomatology. In the latter form of the disease we may have also to deal with the cystic form of the parasite, against which there is as yet no suitable means of testing drugs experimentally. If we compare our results with the results usually obtained in acute amoebic dysentery in man we find reasonable agreement. Thus emetine and chiniofon appear to be the most effective, with stovarsol,

carbarsone, and diodoquin definitely inferior. If, however, we consider the c respective merits of these drugs in chronic amoebiasis, we find them at variance with our experimental results, for against this form of the disease, carbarsone, stovarsol, and diodoquin are undoubtedly of value. The minimal effective therapeutic dose of this type of compound in rat and man differs considerably if the comparison is made on a mg/kg basis. Thus a dose of 1,000 mg/kg, or 5 doses of 500 mg/kg, is required to produce an effect in rats, whereas the dosage used in humans, namely 4 gr twice daily for 10 days (Manson-Bahr, 1945), corresponds to a total dosage of only 80 mg/kg. The validity of such a comparison, however, is questionable, and it is perhaps more reasonable to consider the relationship of the minimal effective therapeutic dose to the toxic dose in the two species. If this is done, the relationship is found to be approximately the same

It is of interest to note the behaviour of diodoquin in our tests. When it was given as a single dose, its therapeutic effect was barely significant, and compared unfavourably with carbarsone and stovarsol. When it was given repeatedly, however, its activity was better demonstrated. The fact that it is poorly absorbed was no doubt responsible for this difference. This compound has been introduced comparatively recently (Hummel, 1939). It does not appear to be much more effective than the other amoebicides (Morton, 1945).

The strain of *E histolytica* used in this series of experiments (isolated in culture from material kindly supplied by Dr A R D Adams) was one which produced infections susceptible to treatment with emetine. Not all strains do so, as has been mentioned in a previous paper (Jones, 1946). It was decided to use an emetine-susceptible strain for comparison as there was no evidence that the other drugs showed similar differences in effectiveness against different strains. The comparison was accordingly carried out under conditions equally favourable to all the test drugs.

STIMMARY

A study has been made of the therapeutic action of emetine, chiniofon, stovarsol, carbarsone, and diodoquin against experimental amoebiasis in rats Emetine and chiniofon appeared to have the widest range of activity Stovarsol, carbarsone, and diodoquin were effective when given in large doses

I thank the following G H Davies, S R Smedley, D Todd, and W A. Whittaker for technical assistance, and Dr O L Davies for advice in the statistical assessment of the results

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THE TECHNIQUE OF TESTING CHEMOTHERAPEUTIC ACTION ON PLASMODIUM GALLINACEUM

BY

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(Received December 21 1945)

During the war *P* gallinaceum and *P* lophurae came into general use for testing the antimalarial action of new compounds. The purpose of the present work, carried out during 1943 and 1944, and withheld from publication owing to wartime restrictions, was to compare different methods of dosage in tests with *P* gallinaceum, and to see how much the answer obtained depended upon the technique employed

In all tests the compounds were judged by their ability to reduce parasitaemia to a low level, little or no account was taken of the power of drugs to sterilize the birds and thus to produce a radical cure of the infection

METHODS

The general plan of the test was based on confidential information received from the American Board for the Co-ordination of Malaria Studies and from Mr, D G Davey, of Imperial Chemical Industries, Ltd

The strain of *P. gallinaceum* used was obtained from the Molteno Institute, Cambridge, and was derived from the original strain introduced into Europe by Brumpt. It was maintained by blood passage in young chickens. The chickens were about 10 days old at the time of inoculation and weighed 60–80 g, they were infected by the intravenous injection of about 10th parasitized cells per chick. In a typical experiment the percentage of parasitized erythrocytes, resulting from this inoculum, was 1 per cent after 2 days, 6 per cent after 3 days, 30 per cent after 4 days, and 50–90 per cent after 5 days, at which time chickens often died, if they survived, the parasites in the blood became less numerous, but many died with exoerythrocytic forms in the brain two weeks after inoculation. The drugs were given by mouth with a syringe and blunt needle. Since most drugs supplied for testing are very limited in amount it was considered that the administration of drugs in the diet (as described by Marshall et al., 1942) would not be practicable. The blood of the infected chicks was examined on the 3rd and 5th days and the percentage of parasitized erythrocytes on the 5th day was determined

To test for the prophylactic activity of drugs, the heads and thoraces of batches of mosquitoes known to contain sporozoites in their salivary glands were ground in saline and the resultant suspension centrifuged lightly to throw down the chitinous parts of the insects. The supernatant was drawn off and made up in a mixture of heparinized chick

plasma and saline, containing at least 50 per cent (v/v) plasma, so that 0.2 c c contained the equivalent of one infected mosquito. This suspension was used to infect chicks by intravenous inoculation of 0.2 c c per chick. Chicks so infected usually exhibited para sites in the peripheral blood 5-7 days after inoculation, and died 6-10 days after infection as a result of massive infection of the endothelial cells of the cerebral capillaries with exoerythrocytic forms. The drugs were administered in the manner described above, dosage being started 2 hours before infection. In both types of experiment the geometrical mean of the responses of the individual birds in a group was taken, this was compared with the geometrical mean of the responses of the group of untreated control chickens

The following salts of quinine, mepacrine (atabrine), and pamaquin (plasmoquin) were used quinine bisulphate containing 59 per cent anhydrous quinine, mepacrine methan sulphonate (quinacrine soluble, May & Baker) containing 65 per cent of mepacrine base (3rd ADD BP 1932, p 15) and the methylene bis-hydroxynaphthoate of 6-methoxy 8 δ diethylamino-α-methylbutyl)-aminoquinoline (pamaquin) containing 45 per cent of base (4th ADD BP 1932, p 24) All amounts of the drugs quoted refer to these salts Sulpha diazine powder was used as the pure substance

Measurement of the blood concentration of the drugs

An attempt was made to measure the levels of quinine, mepacrine, and sulphadiazine in the blood after the different dose schedules employed. In order to facilitate the taking of blood samples, larger chickens were used in these experiments than in the therapeut c experiments.

Quinine —The blood level of quinine at varying periods of time after dosing was measured by a modification of the method of Kelsey and Geiling (1942), kindly devised by Prof C Rimington The estimations were carried out on 0.5 cc blood, drawn from the heart, of 3-4 months old chickens weighing about 800-1,000 g A series of blood samples was taken from each chicken The oxalated blood sample was pipetted into 35 cc distilled water and the blood proteins were digested by the addition of 1 cc 10 per cent sodium hydroxide and heating in a water bath for 15 min The samples vere then extracted with 25 cc of sodium-dried ether containing 5 per cent (v/v) petrol in a 50-cc separating-funnel The ether extract was washed twice with 10 c c N/10 sodium hydroxide and the quinine extracted with 4 cc N/10 aqueous sulphuric acid in three successive volumes of 15 cc, 15 cc, and 1 cc of acid. The acid extract was then made up to 5 cc by the further addition of sulphuric acid and its fluorescence was measured in the Rimington fluorescence comparator (Rimington, 1943) The amount of quining present in the extracts was ascertained by comparison with a standard curve obtained by measuring the fluorescence observed when known amounts of quinine were added to blood and extracted as above

Mepacrine—For the estimations of mepacrine the appropriate doses were given to 800-1,000 g chickens and blood was withdrawn from the heart. Repeated bleedings of the same chickens sometimes led to false readings of the drug level, consequently several birds were placed on the same dose schedule and no bird was bled more than three times. Coagulation of the blood was prevented by potassium oxalate, if the sample had to wait more than 24 hours before examination it was stored at -12° C. The estimations were kindly carried out for us by Major J. Reid of the Royal Army Medical College, he used a modification of Masen's method (1943).

Sulphadiazine —Appropriate doses of sulphadiazine were given to 28-day-old chicks 0.02 cc blood was taken from the leg at intervals, and the concentration of sulphadiazine therein was measured by a modification of the method of Marshall and Cutting (1938). The volume was measured in special micro-pipettes and washed out into 2.5 cc of acid

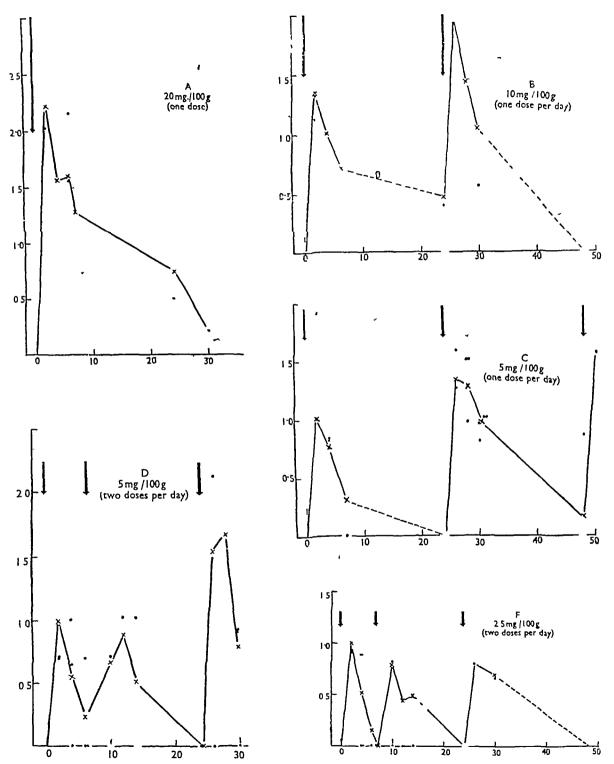


Fig. 1—Blood concentrations of quinine in chicks after oral administration of different dose schedules of quinine bisulphate. Ordinates blood concentrations in mg per litre Abscissae hours after dosage • = levels in individual birds x—x = average levels Arrows indicate dosage

saline (0.85 per cent NaCl in N/50 HCl) The reagents, 0.1 c.c. 0.3 per cent sodium nitrite, 0.1 c.c. 1.5 per cent ammonium sulphamate, and 0.1 c.c. 0.1 per cent naphthyl ethylene diamine dihydrochloride, were then added to 2.5 c.c. of the supernatant, and the intensity of the fully developed colour was measured on a Spekker absorptiometer combined with a spot galvanometer

EXPERIMENTS

TROPHOZOITE-INDUCED INFECTIONS

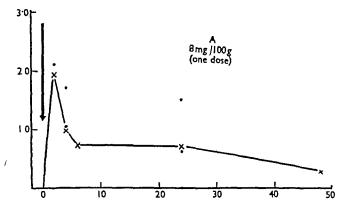
Effect of concentration or dispersion of the dose

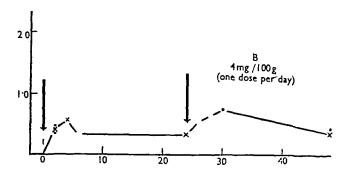
The first investigations were made to determine the effect of concentrating the whole amount of antimalarial compound (quinne, mepacrine, pamaquin, or sulphadiazine) into a single administration or of dispersing it over several days in a series of smaller doses. The total amount administered remained the same, an amount being employed which was about the minimum effective level when given according to the most effective regime. A study was made of the results of the different dose schedules on (A) the resultant blood-concentration, (B) the therapeutic response

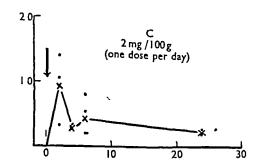
A-Blood concentrations

Quinine—Fig 1 shows the mean curve for the group of birds receiving quinine, it was obtained by taking the average of the blood concentrations at each particular time. Considerable individual variation in the levels was observed in different chickens, particularly in the series 5 mg twice daily for 2 days and 25 mg twice daily for 2 days. The mean curve was only an approximate indication of what might happen in any special instance. On the whole the levels were comparable with those found by Kelsey et al. (1943). The absorption of quinine was rapid the peak in the blood concentration occurring at approximately 2 hours, but the compound soon disappeared from the blood again, so that the period of antimalarial action was presumably brief in most cases. The level attained in the blood was not directly proportional to the size of the dose, since a dose of 25 mg produced a peak mean blood concentration of 10 mg per litre (Fig 1E), while a dose of 20 mg (8 times as great) produced a peak of 21 mg, only twice as high (Fig 1A)

Mepacrine—The blood concentrations of mepacrine are shown in Fig 2, the line being drawn through the mean concentrations at the different periods of sampling. The variations between the different birds were greater than those between the different dose schedules so that from this small number of birds no reliable conclusion could be drawn. On the whole, it appeared that with mepacrine there was a peak in the blood concentration occurring about two hours after the dose, followed by a prolonged plateau at a lower level. This is well exemplified in the curve obtained after the administration of 8 mg per 100 g (Fig 2A). Marshall and Dearborn (1946a) found that in the treatment of P lophurae infections in ducks by mepacrine the therapeutic response was







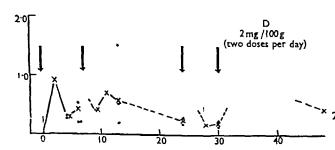
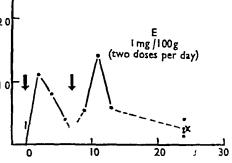
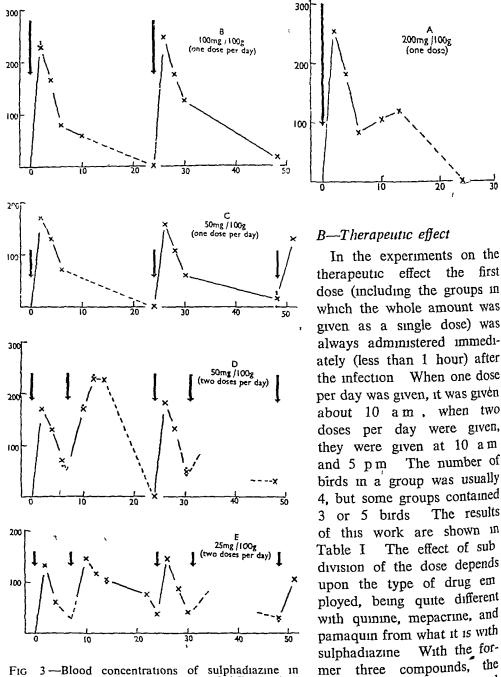


Fig 2—Blood concentrations of mepacrine in Chicks after oral administration of different 10 dose schedules of mepacrine methanesulphonate Ordinates blood concentrations in mg per litre Abscissae hours after dosage / • = levels in individual birds x—x=average levels Arrows indicate dosage

proportional to the dosage rather than to the plasma concentration of the compound Our own limited figures on blood concentrations and on therapeutic response (Table I) are in agreement with their finding

Sulphadiazine —The average blood concentrations obtained when sulphadiazine was given are shown in Fig 3 With one dose per day, even with the largest amount, the drug remained in the blood for less than 24 hours The mean peak level varied with the dose, but not in direct proportion After a dose of 25 mg the peak was 135 mg per litre, after 200 mg (8 times the dose) the peak was 254 mg (Fig 3A and E) effect of subdividing the doses was to prolong the periods in which appreciable amounts of drug were present in the blood and to make them more continuous There was little evidence of an accumulation of sulphadiazine in the blood (Fig 3E)





more the dose is concentrated

the greater the response, and

conversely The drugs are

Fig 3—Blood concentrations of sulphadiazine in chicks after oral administration of different dose schedules of sulphadiazine powder. Ordinates blood concentrations in mg per litre. Abscissae hours after dosage. • = levels in individual birds \(\frac{1}{2} \

TABLE I

THE EFFECT OF DIFFERENT DOSE SCHEDULES OF STANDARD DRUGS ON THE RESPONSE OF

TROPHOZOITE-INDUCED INFECTIONS

		Percentage of cells parasitized on 5th day after different dose s							
Drug	Total dose mg /100 g	Total dose on 1st day	1/2 dose once daily 2 days	1/4 dose twice daily 2 days	1/4 dose once daily 4 days	1/8 dose twice daily 4 days	Untreated controls		
Quinine	20	4 1 1 95 —	0 52 1 18	0 59 1 20 4 18	4 7 24 4 21 9	21 6 17 2	39 3 53 58 2		
Mepacrine	8	0 61 0 1 0 1	1 30 0 1 0 1	52 3 1 8 0 1	48 9 3 7 1 2	47 9 3 1	59 5† 38 5 65 7		
Pamaquin	0 4 0 4 0 6	01 1 06 0 1 0 1	0 1 0 1	5 24* 2 9 1 35 0 1	56 7 70 9 3 09 17 4 0 1	54 7 9 85 2 51 1 2	54 8 58 3 44 63 9 41 4		
Sulphadia- zine	200	37	28 8	38 7	12 6	0 12	39 5		

^{*} Poor test Only 1 chick remained alive

most effective if given in a single large dose or if the dose is subdivided only into two, as the subdivisions get more numerous, less antimalarial effect is shown Apparently the action of these drugs upon the parasites is rapid, a short interval of time being sufficient for it to take place, but on the other hand it is strongly effective only if a certain level of blood concentration is reached eg, with quinine, a level of over 1 μ g per cc, this critical level of concentration is presumably not reached when the dose of drug administered falls below a certain quantity, even if the dose is repeated twice daily for four days sulphadiazine on the other hand, the greatest antimalarial effect is obtained when the administration is spread over the whole four days, subdividing the amount into 8 small doses The administration of the drug in 1-4 doses concentrated into the first two days is non-effective. Even the slight modification of giving a single dose on each of four days, instead of two smaller doses on each of these days, greatly diminishes the antimalarial effect. This class of compound presumably exerts a slower action upon the parasites than quinine, mepacrine, or pamaquin do, and the duration of action is more important than the concentration over any particular short period

Effect of time of starting the treatment

It has been reported from America that some workers begin treatment several hours before infecting the birds, others wait until after the inoculation to begin treatment. The latter procedure is the more convenient in practice as the whole

^{† 6}th day count

batch of chicks can be inoculated without the necessity of identifying each individual in the process, moreover, birds in which the inoculation has been unsatisfactory in any way can easily be discarded and replaced by new ones. An investigation was made in order to discover whether this difference of procedure affected the therapeutic response observed, and the results are shown in Table II. As will be seen, there was no significant difference in the response whether the first dose was given immediately after inoculation, or 5 hours before inoculation. Accordingly, in our routine testing of drugs the first dose is always given almost immediately (less than 1 hour) after inoculation.

TABLE II

THE EFFECT OF THE TIME OF STARTING DOSAGE ON THE RESPONSE OF TROPHOZOITE-INDUCED INFECTIONS

Deve	Total days	Dose given	Percentage of on 5th day whe	Controls	
Drug	Total dose mg /100 g for 14 days		immediately after infection	5 hours before infection	Controls
Quinine	20 24	2 5 mg	10 7 3 02 29	19 14 8 24 9 7 3	38 5 41 4 61 1 44
		3 mg	6	/ 3	
Mepacrine	10	1 25 mg	28 3 0 96 9 01	21 5 1 16 5 52	41 4 44 61 1
Pamaquin	04	0 05 mg 0 075 mg	45 9 21 1 7 94	39 2 28 1 12	38 5 44 63 9
İ	• 1	0 125 mg	1 2	4 59	414
Sulphadiazine	200	25 mg	0 42	0 8*	39 5

Each of the groups of treated chicks contained 4 birds, the groups of controls usually contained 6-10 birds

A further study of the effect of delay in commencing treatment was made by giving the same total amount of mepacrine either all on the last (3rd) day after inoculation or spread out over the previous days. This is a reverse of the dose schedules described in Table I. The results are shown in Table III. The best therapeutic responses were obtained when the drug is concentrated on to the last two days of the treatment. Probably in this experiment the effect of concentrating the dosage was more important than the time of starting it. (On this occasion mepacrine seems to have been less effective than in the experiments of Table I.)

^{*} Started 2 hours before infection in this case as sulphadiazine reaches its peak in the blood 2 hours after oral administration

TABLE III								
THE EFIECT OF DE	LAY IN COMMENCING TR	EATMENT WITH MEPACRINE						

Drug	Schedule	% cells parasitized on 5th day	Control
Mepacrine	6 mg twice daily on the 4th day 3 mg ,, ,, ,, 3rd and 4th days 2 mg. ,, ,, ,, 2nd, 3rd, and 4th days 1 5 mg ,, ,, ,, 1st, 2nd, 3rd, and 4th days	2 62 0 56 18 7 35 2	65 7

In each group the total dose was 12 mg per 100 g Each group contained 4 chicks

SPOROZOITE-INDUCED INFECTIONS

Similar investigations about the effect of concentrating or dispersing the treatment were made with sporozoite-induced infections—i.e., the prophylactic action of the compounds upon the pre-endoerythrocytic forms of the parasites was studied instead of the therapeutic action upon the endoerythrocytic ones (trophozoites, schizonts, etc.) At the time of conducting this work the only compounds known to have a prophylactic action were the sulphonamides, and accordingly the investigations were made using sulphadiazine. Two types of experiment were made. In the first, a given amount of sulphadiazine (200 mg per 100 g.) was either concentrated at the beginning of the infection or dispersed over the first four days, in the second, treatment was more intense (total 800 mg per 100 g.) and it was given either in the first two days or on the 3rd and 4th days after infection. The results of both these investigations are shown in Table IV

In the first type of experiment the data show that the more the treatment was spread out over the first four days the more effective it was, treatment restricted to the first day had little or no effect, even if the total dosage was doubled (i.e., total dose 400 mg). These results with pre-endocrythrocytic stages of the parasite agreed with those obtained concerning the action of sulphadiazine upon the endocrythrocytic forms, they support the view that the antimalarial action of sulphadiazine is of such a type that duration of exposure is more important than intensity

The second part of these experiments concerns the sensitivity of the different stages of the parasite to sulphadiazine. Treatment restricted to the first day had little or no action, apparently the sporozoites in their original form or in their early development after inoculation were not much affected by the compound during a single day. Treatment given on the 3rd and 4th days was slightly more effective in delaying the development of patent infection than that given on the 1st and 2nd days, but much of this effect was due to the postponement of treatment, and if the interval is measured between the onset of the patent infection and the end of treatment, there was little real difference between the degree of parasitaemia observed in the two groups. However, ultimate survival was much

, TABLE IV

EFFECT OF DIFFERENT DOSE SCHEDULES AND OF TIME OF DOSAGE OF SULPHADIAZINE ON SPOROZOITE INFECTIONS

	Total dose	Individual doses	Days of		Percentag	ge of cells	parasıtız	ed on	day	Remarks
	mg /100 g	per 100 g	treatment	8	9	10	11	12	13	Kemarks
	200	*200 mg once daily	1st		24 6 2 D EEP+		23 1 D EEF+			
	200	100 mg once daily	1st, 2nd		1 2	2 D EEF+	49 3 2 D			
First part of experiment	200	50 mg once daily	1st, 2nd, 3rd, 4th		Less than 0 1		16		14 1 2 D EEF+	2 chicks died on 17th day
	200	*50 mg twice daily	1st, 2nd		Less than 0 1		0 66		14 9	3 chicks died on 166 day EEF+
	200	*25 mg twice daily	1st, 2nd, 3rd, 4th		Less than 0 1		Less than 01			On 15th day 512 1 D EEF+ 1 chick died on 16th day 1 chick sur vived
	Control	*	_		45 5 2 D		1 D			
	200	*200 mg	1st	10 2	1 D	48 2 D EEF+	1 D			
	200	25 mg twice daily	1st, 2nd 3rd, 4th	Less than 0 1		Less than 0 1	0 4			2 chicks failed to show parasit.ema. 2 chicks died on 17th day EEF+
	Control			8 03 1 D	1 D EEF+	32 2	2 D			
1	800	200 mg twice daily	1st, 2nd	Less than 01		1 2		69	2 D	2 chicks died on 14th day EEF+
	400	200 mg twice daily	1st	43	1 D EEF+	11 6	1 D	1 D		1 chick died on 14th day
ond part	Control	*			45 5 2 D		1 D		,	
Second	800	*200 mg twice daily	1st 2nd	Less than 0 1		3 1	1 D EEF+		42 5 2 D	
	800	200 mg twice daily	3rd, 4th	Less than 0 1		Less than 0 1			24 3	1 chick died on 17th day EEF+ chicks survived
	Control			8 03 1 D	1 D EEF+	32 2	2 D			

The inoculum used in these experiments contained the approximate equivalent of 1 infected mosquito per chick. The groups marked with an asterisk contained 3 chicks, the others contained 4. The '1st day dose was given 2 hours before infection with sporozoites. D, Chick died EEF— Exoerythrocytic forms found in the brain post mortem.

better in the group treated on the 3rd and 4th days. It is noteworthy that (with the possible exception of 2 chicks) sulphadiazine in these doses failed to sterilize any of the birds, it only delayed the multiplication of the parasites for a shorter or longer period. In this respect its action upon pre-endoerythrocytic forms was indistinguishable from that upon endoerythrocytic forms. The results thus suggest that the antimalarial action of sulphadiazine is exerted both on pre-endoerythrocytic forms of P gallinaceum (cryptozoites, etc.) and on endoerythrocytic forms (trophozoites and schizonts), that this action tends to be plasmodiostatic rather than plasmodiocidal, and that its effectiveness depends upon the duration of exposure rather than upon the intensity

DISCUSSION

It has been shown that under the experimental conditions described the maximum effect of a given small quantity of quinine, mepacrine, or pamaquin upon the trophozoites is obtained when the treatment is concentrated into one or two days. The greatest effect of a given quantity of sulphadiazine upon either trophozoites or upon pre-endoerythrocytic forms is obtained when the treatment is spread out over the whole period of four days. With the former drugs, intensity of action seems more important than duration, with sulphadiazine, duration is more important than intensity. In the treatment of trophozoite-induced infections there is no significant difference in the response observed whether the first dose is given 5 hours before inoculation or immediately after it. The action of sulphadiazine is exerted both on pre-endoerythrocytic forms of P gallinaceum and on endoerythrocytic ones

A comparison has been made by Marshall and Dearborn (1946b) of the single-daily-dose and of the drug-diet methods of treatment in bird-malaria (*P lophurae* in ducks) in relation to the therapeutic activity of numerous compounds, as found above, they showed that the relative activities of the different compounds are considerably affected by the concentration or dispersion of the dosage

These results may now be considered in relation to the devising of routine tests of the antimalarial action of new compounds. If the compound were given in a single dose (as is customary in experiments with mouse trypanosomiasis) the action of quinine, etc., would be manifested while that of sulphadiazine would probably be missed. The maximum dose which can be tolerated depends upon the toxicity of the compound, and this is usually such that a series of doses spread over four days is much less injurious than a large dose on a single occasion. Accordingly the usually accepted regime of treatment spread over four days has much to recommend it since (1) it provides sufficient duration for the slower acting compounds such as sulphadiazine, (2) it minimizes toxicity and allows larger amounts of the compound to be given, and (3) it is not unduly laborious to administer. No experiments have been made in this work using schedules extending over more than four days as a routine measure such schedules would appear

to be more wasteful of labour and of material (which is often scarce) while offering insufficient compensating advantage The routine tests carried out in this laboratory are therefore based on treatment lasting four days. The general procedure has been described above in the section upon methods tions induced by trophozoites the response is read as the percentage of parasitized erythrocytes on the fifth day, and the geometrical mean for the group of birds is compared with that for the group of untreated controls. If a compound is found to be active in the maximum tolerated dose, the dose is reduced in subse quent tests until the lowest dose is found which reduces the percentage of parasitized cells to about one or two This is considered to be the approximate minimum effective (therapeutic) dose For infections induced by sporo zoites, the chief criteria of the activity of a drug are complete suppression of infection or a delay in the appearance of parasites in the peripheral circulation, and prolongation of the life of the chicken or recovery from the infection quantitative determination of the activity, measurement is made of the minimum effective (prophylactic) dose which prevents the percentage of parasitized cells being greater than 1-2 on the 7th to 9th day when the infection has reached a high level in the controls, no compound tested to date in this laboratory has been sufficiently effective for its activity to be expressed as the minimum dose which completely prevents infection. The antimalarial activity, therapeutic or prophylactic, as expressed by these minimum effective doses, is compared with the toxicity as determined by the maximum tolerated dose for mice, when administered orally twice daily for four days Since the drugs are designed for use in man, the toxicity for mammals is more significant than that for birds, even though the tests for antimalarial potency are carried out in chickens

SUMMARY

The methods used in the authors' laboratory for testing the antimalarial action of drugs upon infections of P gallinaceum induced by trophozoites and sporo zoites are described

A given amount of quinine, mepacrine, and pamaquin exerts the maximum effect on trophozoite-induced infections of P gallinaceum if it is concentrated into the first day or first two days of treatment, with these compounds intensity of action is more important than duration. A given amount of sulphadiazine produces the maximum effect upon trophozoite- or sporozoite-induced infections if it is dispersed over all the four days of treatment, with this compound duration of action is more important than intensity. The action of sulphadiazine is exerted both on the pre-endoerythrocytic forms of P gallinaceum (cryptozoites, etc.) and on the endoerythrocytic forms (trophozoites, etc.)

In the test on trophozoite-induced infections there is no significant difference in the response whether the first dose is given immediately after the inoculation or 5 hours before it

The blood concentration curves of quinine, mepacrine, and sulphadiazine on the different dose schedules were determined. Increasing the dose eight times increases the peak concentration in the blood only about twice

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THE TOXICITY OF ALKYL FLUOROPHOSPHONATES IN MAN AND ANIMALS

BY

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In May, 1940, we prepared the dimethyl and diethyl esters of fluoro phosphonic acid and tested their effects on animals as lethal inhalants. When, about a year later, McCombie and Saunders prepared the disopropyl ester, we found it I an even more powerful effect than the dimethyl and diethyl compounds

$$RO$$
 P O $R = CH_3$, C_2H_5 or $(CH_3)_4CH$ $-$

dialkyl fluorophosphonate

The present paper deals with this early and first systematic study of the toxicity of the fluorophosphonates which are now widely used for physiological experiments and clinical trials. When the toxic action was discovered it was not possible for us to publish the results, which were, however, circulated as a report to the Ministry of Supply (Adrian, Kilby, and Kilby, 1940)

The preparation of the fluorophosphonates and this study of their toxic properties was prompted by an observation of Lange and Krueger (1932). They had prepared the dimethyl and diethyl compounds and had stated at the end of their communication that inhalation for a few minutes might lead to difficulty in breathing, to disturbance of vision, hypersensitivity to light, and even to loss of consciousness.

METHODS

The dimethyl and diethyl fluorophosphonates used in these experiments were prepared by the method described by Lange and Krueger, and the disopropyl ester was kindly supplied by Dr H McCombie, Dr B C Saunders and their research team, who made it by the method announced in a preliminary communication (McCombie and Saunders, 1946)

The action and toxicity of fluorophosphonates were studied in man by inhalation and in animals by inhalation as well as by intravenous or subcutaneous injection

Inhalation in man

An approximately cubical steel-framed glass chamber of 10 cu.m capacity was used. The desired concentration of ester vapour was obtained by dissolving the calculated

weight of the compound in about 20 cc of ether and spraying it into the chamber by means of an atomizer worked by compressed air, mixing being achieved by three electric fans. After about 30 sec, the subjects, one to four in number, who had been waiting in an air-lock, walked into the chamber and quickly shut the door behind them. At the end of the exposure, the subjects left through the air lock, and the chamber was cleared by a large suction fan. In the calculation of the concentration (e.g. 1 in 100,000) it is assumed that the ester is completely volatilized and that the gram-molecular weight occupies a volume of 22.4 litres.

Inhalation in animals

Static method—A wood-framed glass chamber of A batch of animals (e g, 3 rabbits, 4 guinea-pigs, 6 rats wire cages, the chamber sealed and the calculated a in 20 c c of ether sprayed into it. The expositive material had been dispersed, an operation Mixing was achieved by an electric fan in exhausting fan was switched on, the chambe— operators wearing service respirators. The animals were

rapacity was used
y placed inside in
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operators wearing service respirators. The animals were removed to another room and kept under observation. Post-mortem examinations were made on animals that died

Constant-flow technique -An apparatus was constructed containing an all-glass exposure chamber of about a litre capacity fitted with ground glass joints, in which either one rat or four mice could be placed. A constant stream of air, at a known rate of flow, was passed through this chamber, either by using compressed air which was passed through a gas-meter in series with the chamber or, in other experiments, by suction, measured amounts of water being run out of a 20-litre aspirator All or part of the air stream could be passed through a bubbler containing dissopropyl fluorophosphonate, which was weighed before and after the experiment to determine the amount of ester volatilized The rate of volatilization was also controlled by varying the temperature of the water-bath in which the bubbler was immersed An identical bubbler containing sulphuric acid was fitted in parallel, and in an exposure pure air was passed through the chamber until conditions were steady and the animal calm, and then by means of a three-way tap the air stream was caused to pass through the ester for the desired time. In this manner it was possible to make 1-min exposures, because the desired concentration was rapidly attained at the beginning of the exposure, and the ester quickly swept out at the end part of the total air stream was passed through the bubblers, flow-meters were included in the circuit so that the air streams could be adjusted to give approximately the desired concentration of ester The two air streams were allowed to mix in a suitable compartment before entering the exposure chamber The bubblers, mixers and exposure chamber were connected by ground glass joints When necessary, the gases leaving the exposure chamber were passed through towers containing activated charcoal or through service respirator canisters in order to remove the ester vapours

RESULTS

Inhalation in man

The inhalation of air containing low concentrations of dimethyl, diethyl, or disopropyl fluorophosphonate leads to effects qualitatively similar for all three esters, but varying in intensity with the nature of the ester dispersed, its concentration, and the time of exposure. Within a minute or so a feeling of tightness of the throat is noticed and respiration becomes slightly more laboured

A few minutes later the pupils constrict to pin-point size and remain so for a long period, this causes the subject to experience the sensation that the room has dimmed, as though the sky had suddenly become heavily overcast. After a few hours, reading becomes almost impossible unless the book is held only a few inches from the eyes, at a distance of about 18 in print appears blurred. This effect must be due to a strong constriction of the ciliary muscles. The dissopropyl ester has the most powerful action of the three compounds. When two subjects were exposed for 3 min to a nominal concentration of 1 in 100,000 (82 mg/cum) of this material, a tightness of the throat and slight difficulty in inspiration occurred during the exposure, and about 10 min later the pupils constricted to pin-point size and remained so for days. The miosis subsided after about a week in the younger subject (28 years) and after two or three days in the elder (60 years). After about 24 hours there was eye-ache and headache which persisted for a day or two

Inhalation in animals

Animals were exposed for longer periods and to higher concentrations than those used with man, and severe symptoms were produced, death frequently resulting. The effects were approximately the same in all species examined and with each of the three compounds. There was excessive salivation, nasal discharge, lacrimation and frequently pupil constriction, respiratory distress accompanied by intense gasping movements, and, in severely affected animals, convulsions leading to death, which usually occurred within about half an hour of the beginning of the 10-min exposure, and sometimes actually during the exposure. The rapidity of action of these materials as lethal inhalants is noteworthy and the majority of animals that survived half an hour usually made a complete recovery, although a few deaths occurred up to 24 hours

The mortalities produced in rabbits, guinea-pigs, rats, and mice by 10-min exposures to various nominal concentrations of the three compounds are shown in Table I For each experiment 3 rabbits, 4 guinea-pigs, 4 or 6 rats, and 4 or 10 mice were exposed in a static chamber. Of the four animal species, it will be seen that rabbits are the least and mice the most susceptible

TABLE I

DEATHS RESULTING FROM 10-MIN EXPOSURE OF SMALL ANIMALS TO VARIOUS CONCENTRATIONS OF FLUOROPHOSPHONIC ESTERS

Compound	Methyl ester				Ethyl este	<i>Iso</i> prop	yl ester	
Concentration	1/5 000	1/10,000	1/20 000	1/5,000	1/10,000	1/20 000	1/10 000	1/20,000
Rabbits Guinea-pigs Rats Mice	0/3 3/4 4/4 4/4	,0/3 0/4 4/6 10/10	0/3 0/4 1/4 4/4	0/3 2/4 4/4 4/4	0/3 0/4 0/6 10/10	0/3 0/4 1/4 0/4	2/3 0/4 6/6 10/10	0'3 0 4 4'6 3'10

A more detailed study was made of the toxicity of the disopropyl ester for rats and mice, the concentration necessary to kill 50 per cent of the animals exposed (LC50) was determined for each of five constant exposure times (1, 2, 5, 10, and 30 min) by the constant-flow technique A convenient measure of the toxicity of a lethal inhalant is obtained by the product of the LC50 and the time of exposure in minutes (t) This value is referred to in the present paper Ideally it should be a constant and independent of the exposure as the LCt50 time, but if detoxification takes place during exposure LCt50 should increase greatly during long exposure to a low concentration, this is observed, for instance, when hydrogen cyanide is used as a lethal inhalant. The small changes observed in the values of LCt50 in our experiments with fluorophosphonates, however, were probably not statistically significant, but a minimum value of the LCt50 at 5-10 min might be indicated With the static method an increase in LC150 with longer exposures may indicate that the concentration of toxic agent is falling off, but this effect can be eliminated by the constant-flow technique, in which a constant concentration is maintained by replacement. The LCt50 values for rats using 10- and 30-min exposures were determined by both static and constant-flow methods, and almost identical values were obtained, indicating that no appreciable decay of concentration occurred in the static method up to 30 min, so that the nominal concentrations employed in the static method used to obtain the data in Table I are probably fairly close to the true concentration

Estimates of the LC50 and LC150 values for rats and mice are shown in Table II These estimates were obtained, by graphical interpolation, from the results of experiments in which at least 4 animals were used to determine each point on the mortality-concentration curves, in all, 197 rats and 390 mice were used

TABLE II

LC50 AND LC150 VALUES FOR RATS AND MICE EXPOSED TO DI150 PROPYL FLUOROPHOSPHONATE

CONSTANT-FLOW TECHNIQUE

	i	Deaths	within 2 hr	Deaths within 48 hr		
Anımal	Exposure time min	LC50 mg/cu m	LC150 mg/cu m/min	LC50 mg/cu m	LC150 mg /cu m /min	
Rats	1 2 5 10 30 1 2 5 10 30	4 200 2 000 700 360 180 5,000 2,650 750 440 185	4 200 4 000 3 500 3 600 5 400 5 000 5,300 3,750 4 400 5,550	4 200 1,800 570 280 150 4,000 1,900 540 350 150	4,200 3,600 2,850 2,800 4,500 4 000 3 800 2 700 3 500 4,500	

A more detailed analysis of the survival period of these animals shows that 55 per cent of the rats and 53 per cent of the mice had died within the first 2 hours after exposure, during the next 48 hours a further 15 per cent of the rats and 22 per cent of the mice died, there were no further deaths among the rats and only 1 per cent among the mice, 30 per cent of the rats and 23 per cent of the mice surviving. The rapid lethal action of fluorophosphonates becomes even more evident if the deaths occurring during the first two hours are grouped in half-hour intervals, if for this calculation the deaths after 30-min exposure are omitted, it is found that the majority of animals died within the first half-hour after exposure. Of the 108 rats dying within the first two hours, 95 died within the first, 10 within the second, and 3 within the third half-hour period after the beginning of exposure, the corresponding figures for 207 mice dying during the first two hours were 183, 10, 9, and 8 mice respectively for the four half-hour intervals

Injection into animals

After injection of a solution of the disopropyl ester in normal saline into the ear vein of rabbits there was excessive salivation, muscular twitchings, and loss of muscular co-ordination, sometimes urination and defaecation and usually convulsions prior to death. The pulse-rate was slowed like the respiratory-rate, but respiration ceased before the heart stopped beating. There was constriction of the pupils, commencing 2–5 min after injection. The LD50 determined in a small number of rabbits was 0.5–0.75 mg/kg.

The LD50 for mice for subcutaneous injection of the dissopropyl ester dissolved in normal saline was 4 mg/kg, determined by graphical interpolation of the results of injections into batches of mice over a suitable dose range

Atropine

Since it had been found (see Adrian, Feldberg and Kilby, 1946, 1947) that fluorophosphonates were extremely strong inhibitors of cholinesterase, experiments were made to determine whether atropine would be an effective antidote

In rabbits intravenous injections of atropine were made either before or immediately after intravenous injection of a lethal dose of disopropyl fluorophosphonate. When atropine was injected after the ester it was incapable of saving life, but when it was injected before the ester it appeared to reduce the death rate. For instance, when atropine (in doses between 4 and 50 mg/kg) was injected immediately after a lethal dose of disopropyl fluorophosphonate (1 mg/kg) the sole effect was to alleviate the severity of the symptoms and postpone death for a short time. This effect of atropine occurred only if the atropine was given immediately after the fluorophosphonate, if given only a few minutes later atropine had no alleviating effects whatever. When, on the other hand, atropine (10 mg per kg) was given intravenously 10 min before a lethal intravenous dose of fluorophosphonate (1 mg/kg) the appearance of symptoms was not only delayed and their severity reduced, but the lives of some of the animals were actually saved, for instance, out of five rabbits so treated, three survived and the other two died only after 1½ to 3½ days, whereas all five control rabbits, given the fluorophosphonate without atropine, died within 45 min

DISCUSSION

The toxic effects of the fluorophosphonates resemble in many respects those of eserine and prostigmine and are probably due to the anti-cholinesterase activity which these esters have been shown to exhibit (Adrian, Feldberg, and Kilby, 1946, 1947) Parasympathomimetic effects are very pronounced the eye effects, the excessive salivation and lacrimation, and the slowing of the heart may easily be explained as being due to accumulation of acetylcholme released It is thus not surprising that these effects from the parasympathetic endings are alleviated by atropine. In addition, the fluorophosphonates appear to have a definite "nicotine-like" action on skeletal muscle and on the central nervous The excitatory effects of fluorophosphonates on these structures are less pronounced, and the paralysing effects predominate The fluorophosphonates share this predominance of a paralysing action with other anti-cholinesterases, and the problem of why under certain conditions some inhibitors of cholinesterase are mainly excitatory and others mainly depressant has never been satisfactorily explained (For review see Feldberg, 1945)

Death from fluorophosphonate poisoning probably results from respiratory tailure, partly owing to obstruction of bronchioles, but mainly to paralysis of the respiratory centre, in this way, too, the fluorophosphonates resemble other anti-cholinesterases. However, no detailed analysis has been made of the exact nature of the cause of death

SUMMARY

- Inhalation of fluorophosphonates in man and animals leads to respiratory distress, pupillary constriction, and spasm of accommodation. In animals, inhalation in higher concentrations than those used in man causes in addition excessive salivation, lacrimation, convulsions, and death, probably owing to respiratory failure. Similar effects are observed in animals on intravenous injection of fluorophosphonates.
- 2 The percentage of deaths among small animals after 10 min exposure to various concentrations of dimethyl, diethyl, and dissopropyl fluorophosphonates is recorded. The concentration of dissopropyl fluorophosphonate which will kill 50 per cent of rats or mice exposed (LC50) was found for 1, 2, 5, 10, and 30 min exposures. The product of the LC50 and the time of exposure in minutes (the LCt50) was found to vary between 2,700 and 4,500 mg/cu m/min. The LCt50 showed a minimum for 5 to 10 min exposures.
- 3 Death, if it occurred, usually took place within the first half-hour after exposure
- 4 The LD50 of dusopropyl fluorophosphonate is about 4 mg/kg for subcutaneous injection in mice and 05-075 mg/kg for intravenous injection in rabbits

5 Atropine given before the fluorophosphonate alleviated the severity of symptoms and reduced the death rate, but had little effect if given afterwards

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THE MODE OF ACTION OF MYANESIN

BY

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Berger and Bradley (1946, 1947) have shown that α β -dihydroxy- γ -(2-methylphenoxy)-propane ("myanesin") produces muscular relaxation and paralysis in experimental animals. The effects of the drug are of particular interest because paralysing doses of invanesin do not cause arrest of respiration. In this respect myanesin differs from curare and similar muscle-relaxing agents, which do not produce paralysis without simultaneous respiratory depression or arrest. It was therefore of interest to examine in greater detail the mechanism by which the effects of myanesin are produced. This report describes certain aspects of the action of the drug on voluntary muscle, the myoneural junction, peripheral nerves, and the central nervous system

Action on the isolated voluntary muscle

The action of myanesin was investigated on the isolated rectus abdominis of the frog (*R temporaria*) during November and December The muscle was suspended in oxygenated Ringer's solution and arranged for recording the contractions on a kymograph Concentrations of drugs were expressed as final concentrations in contact with the muscle Myanesin at 1 in 750 or higher dilutions did not cause any effects Stronger solutions such as 1 in 500 produced a slow contracture, which began about 10 minutes after the addition of the drug and gradually increased until about 1 hour later the muscle had shortened by about one quarter of the maximum possible contraction. At this stage the muscle still responded to electrical stimulation

The effect of myanesin on the contraction produced by acetylcholine was also investigated. Acetylcholine, 1 100,000, produced almost maximum contraction, after 2 similar contractions had been produced, myanesin or other drugs were added and allowed to remain in contact with the muscle for 10 minutes. The muscle was then washed and the response to acetylcholine retested at suitable intervals until contractions were obtained similar to those before application of the drug.

Table I gives the reduction of the contraction expressed as the percentage of the contraction before administration of the drug Myanesin 1 in 1,000 reduced the response to acetylcholine to about one half and 1 in 750 to about one third Stronger solutions of myanesin were not used because they affected the muscle itself Tubocurarine chloride and procaine hydrochloride, when similarly examined, were found to exert a very much stronger effect than myanesin The

TABLE I

THE ANTAGONISTIC EFFECT OF MYANESIN AND TUBOCURARINE ON CONTRACTIONS OF THE FROG'S RECTUS ABDOMINIS

Contractions were produced by acetylcholine 1 in 100,000

Drug	Dilution	Reduction of contraction as per cent of original contraction
Myanesin	1 2,000 1 1,000 1 750	25 53 68
Tubocurarine chloride	1 1,000,000 1 750,000 1 500,000 1 100,000	36 46 60 93

concentrations of tubocurarine chloride, procaine, and myanesin causing approximately 50 per cent reduction in the response to acetylcholine were 1 in 700,000, 1 in 6,000, and 1 in 1,000 respectively

The experiments show that myanesin in high dilution does not antagonize the action of acetylcholine on voluntary muscle. It is therefore likely that myanesin produces its effects by a mechanism different from that of curare Although myanesin and procaine are local anaesthetics of almost equal potency, procaine antagonizes the effects of acetylcholine more strongly than myanesin. This observation suggests that the curare-like action of local anaesthetics is an independent property of such drugs and may have little relation to their paralysing effects on nerve endings or nerve trunks.

Effect on peripheral nerves

The action of myanesin on the motor nerve was examined on a simple muscle nerve preparation of the frog. The sciatic nerve was immersed in a solution of the drug Excitability was tested at the cut end of the nerve at 1 minute intervals with galvanic current from an induction coil. The time after which contraction of the muscle was abolished was noted. The action on sensory nerves was examined by the plexus anaesthesia method in frogs and the intracutaneous weal method in guinea-pigs as described by Bulbring and Wajda (1945). Cocaine or procaine were used as standards of comparison. All drugs were used at three dose levels.

TABLE II

LOCAL ANAESTHETIC POTENCY OF MYANESIN EXPRESSED IN TERMS OF PROCAINE AND COCAINE

Method	Number of animals used with myanesin	Number of animals used with procaine	Potency as percentage of that of procaine	Number of animals used with cocaine	Potency as percentage of that of cocaine
Motor nerve (frog) Sensorv nerve (frog) Intracutaneous weal (guin pig)	12 12 18	12 12 18	98 96 69	12 12 —	35 39 —
	1	1			

The results are summarized in Table II Myanesin had about the same potency as procaine and one third that of cocaine when tested on the motor and sensory nerve of the frog When examined by the intracutaneous weal method in guinea-pigs, myanesin had about two thirds of the activity of procaine The relatively weak local anaesthetic action of myanesin suggests that the paralysing effect of the drug is not due to a direct action on peripheral nerves

The curare-like action

It has been shown previously that myanesin in large and nearly lethal doses can produce paralysis of the muscle to indirect but not to direct stimulation. This effect may be due either to a curare-like action at the myoneural junction or to a more central paralysis of the nerve-endings (Berger and Bradley, 1946). Further experiments were carried out to ascertain the importance of this effect in myanesin paralysis.

- (a) Experiments on cats —Chloralosed or decerebrated cats were arranged for registration of contractions of the gastrocnemius muscle. The muscle was stimulated alternately directly and indirectly at 10 seconds intervals by single induction shocks. Intravenous injection of myanesin in doses up to 150 mg did not effect the response to direct or indirect stimulation in any way. Tubocurarine chloride (0.5 mg) abolished the response of the muscle to indirect stimulation but hardly influenced the response to direct stimulation. The experiments show that myanesin injected into cats in doses of 150 mg does not possess curare-like action.
- (b) Experiments on mice—White mice weighing 18 to 22 grams were injected intraperitoneally with myanesin, cocaine, or procaine. After 10 minutes they were anaesthetized with ether and decerebrated. The sciatic nerve was then exposed in the gluteal region and cut. Fifteen and thirty minutes after injection of the drug, the peripheral end of the nerve was stimulated with faradic current of increasing voltage and the presence or absence of contraction of the muscles of the leg was noted

Myanesin in doses of 300 mg per kg (i.e., about 50 per cent of the LD50) did not cause paralysis of the muscle to indirect stimulation. Larger doses such as 500 mg per kg (80 per cent of the LD50) made even the strongest stimulation of the nerve ineffective after 30 min, but did not significantly alter the threshold to direct stimulation The disappearance of the response to indirect stimulation could be caused by a depression of neuromuscular transmission or by paralysis of the nerve It was of interest to ascertain whether other local anaesthetics. such as cocaine or procaine, could paralyse motor nerves when administered in very large doses Cocame in doses as large as 100 mg per kg (LD75) did not abolish or impair the response to indirect stimulation Similar results were obtained with procaine It does not appear possible to produce paralysis of peripheral nerves by the systemic administration of local anaesthetics such as cocaine or procaine It is therefore unlikely that paralysis to indirect stimulation produced by large doses of myanesin could be due to a paralysing action on the nerve, particularly as myanesin is a less potent local anaesthetic than cocaine Myanesin may have an effect on the nerve because of a selective affinity of the

nerve tissue for the drug, but no evidence in favour of this assumption is available. It appears more likely that myanesin can block neuromuscular transmission when administered in very large doses. This curare-like effect does not play any part in the production of reversible muscular paralysis with smaller doses because under such conditions paralysis to indirect stimulation was never observed.

The myanesin-strychnine antagonism

It has been shown previously that myanesin can antagonize the actions of strychnine (Berger and Bradley, 1946) In those experiments the drugs were administered subcutaneously in 25 per cent gum acacia solution. To measure the antagonistic effect more accurately a series of experiments was carried out in which the drugs were administered intravenously. With this mode of administration, variations due to differences in the speed of absorption were eliminated

White mice weighing 18 to 22 grams were used. Injections were made into the tail vein at a rate of 0.3 c.c. per minute. The convulsant and myanesin were injected together in a volume of 0.4 c.c. per 20 grams body weight.

The minimal lethal doses (MLD), approximately equal to the LD80, of strychnine sulphate and leptazol were 0.43 and 100 mg per kg respectively. The median lethal dose (LD50) of myanesin was 320 mg per kg

Mice were injected with single minimal lethal doses of strychnine or leptazol, or multiples thereof. Myanesin was administered simultaneously and, for each dose of the convulsant, doses of myanesin were found which protected some of the animals from death. From these values the dose of myanesin protecting 50 per cent of animals was found graphically by plotting the probits of the percentage mortality against the log doses.

TABLE III STRYCHNINF-MYANESIN ANTAGONISM AFTER SIMULTANEOUS INTRAVENOUS ADMINISTRATION TO MICE

Strychnine dose as multiple of MLD	Myanesin		Ratio to number injected of number		Dose of myanesin protecting
	mg /kg	fraction of LD50	convulsed	died	50% of mice mg/kg
1 1 1	40 20 10	1/8 1/16 1/32	35/35 0/10 2/10 9/10	30/35 0/10 2/10 6/10	12
2 2 2 2	80 40 20	1/4 1/8 1/16	0/20 17/20 10/10	0/20 7/20 9/10	34
3 3 3	320 160 80	1 1/2 1/4	0/10 6/10 10/10	0/10 2/10 5/10	80
4 4 4	320 160 80	I 1/2 1/4	7/20 20/20 10/10	12/20 10/20 10/10	160

The antagonistic action of myanesin against leptazol was relatively weak About one quarter of the LD50 of myanesin protected 50 per cent of the animals, against 1 MLD of leptazol. The effect of myanesin in maintaining life was greater than its power to prevent the occurrence of convulsions. Protection against 2 MLD of leptazol could not be obtained even when administered together with one LD50 dose of myanesin.

The antagonistic action of myanesin to the effects of strychnine was well marked. Animals could be protected from the effects of one MLD dose of strychnine by as little as one thirtieth of the LD50 of myanesin, and proportionally larger doses were able to antagonize larger doses of strychnine (Table III)

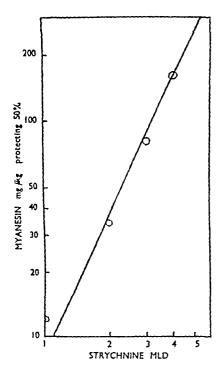


FIG 1—Antagonism of myanesin and strychnine Abscissae Dose of strychnine as multiple of MLD \ Ordinates Dose of myanesin protecting 50 per cent of animals

When the median protective dose of myanesin was plotted against the dose of strychnine, expressed in terms of MLD, both values being plotted on a logarithmic scale, an approximately straight line was obtained (Fig 1)

In suitable dosage myanesin antagonized all the effects of strychnine, and animals to which balanced mixtures of the two drugs had been administered appeared quite normal. The myanesin-strychnine antagonism was more complete than the hexobarbitone-strychnine antagonism, in the latter it was not possible to find a balanced mixture, because doses of hexobarbitone which protected mice from convulsions and death caused a depression of greater duration and intensity in the presence of strychnine than in its absence

Strychnine causes an increase of the reflex irritability of the spinal cord which may result in simultaneous contraction of all skeletal muscles if sufficiently large doses are given Myanesin selectively antagonized the effects of strychnine in doses which by themselves did not produce any effects. It may therefore be inferred that myanesin decreased reflex hyper-

excitability and prevented the passage of abnormal excitatory impulses through the reflex arcs

Effect on tetanus

The effect of myanesin on experimental tetanus was examined in white mice. Ten micrograms of crude tetanus toxin were injected into the neighbourhood of the sciatic nerve high in the thigh. Local tetanus was apparent about 12 hours later and all animals died of generalized tetanus in about 3 days. Myanesin was administered intraperitoneally or subcutaneously at various times after tetanic spasms became apparent. In doses of

150 to 200 mg per kg it completely abolished the spasm in all stages of the disease. The board-like rigidity of the extremities gave place to complete flaccidity a few minutes after injection of the drug. Myanesin in these doses did not cause depression of respiration or any other untoward effects and poisoned animals under the influence of the drug were indistinguishable from control animals to which myanesin only had been given. The effect of the drug lasted for about 30 min and was followed by a gradual reappearance of tetanus. Repeated doses of myanesin caused similar effects and regularly released the spasm. The impression was gained that the life of the animal could have been saved by continuous or frequently repeated administration of the drug.

The action of tetanus toxin on the central nervous system is similar to that of strychnine. The fact that myanesin can re-establish reciprocal innervation in conditions produced by these two different agents gives rise to the hope that it may also influence pathological excitatory innervation caused by degenerative processes of the central nervous system.

Effect on knee jerk

The effect of myanesin on the knee jerk was studied in cats. The animals were anaesthetized with chloralose intravenously and arranged for recording of the knee jerk as described by Schweitzer and Wright (1937). The limb was allowed to hang freely in order to facilitate observation of alteration in muscle tone. The jerk as a rule was elicited every 10 seconds.

Myanesin did not abolish the knee jerk of healthy cats. Intravenous doses ranging from 20 to 150 mg per cat either did not cause any alteration or somewhat reduced the height of the myographic record (Fig. 2), the flexion at the knee joint was usually increased,

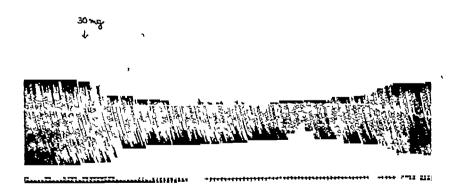


Fig 2—Cat 28 kg Chloralose (0 08 g per kg) Record of knee jerk Time intervals 30 seconds At arrow 30 mg myanesin slowly injected intravenously

signifying a decrease in the tonus in the muscles, but complete inhibition of the jerk was not observed

Some of the cats used in the experiments showed a very lively reflex followed by clonus. Exaggerated reflex excitability and tremors were also observed in certain animals under light chloralose anaesthesia (0.05 g. chloralose per kg.). Myanesin had a definite

effect on the knee jerk of these animals (Fig 3A and B and Fig 4) It inhibited clonus and tremors, abolished the irregular responses to patellar stimulation and reduced the knee jerk to its usual size. These effects could be produced with small doses of myanesin (20 to 30 mg per 3 lg cat). The effect set in almost immediately and lasted for about 20 minutes and sometimes longer.

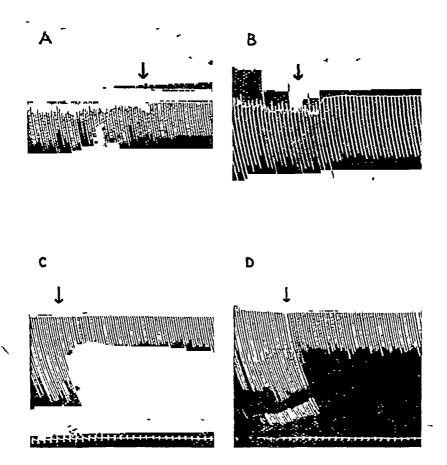


Fig 3—Records of knee jerk in cats weighing 2 8-3 1 kg

Jerk elicited every 10 seconds

Drugs injected intravenously

A Chloralose 0 05 g per kg, myanesin 50 mg

B Chloralose 0 06 g per kg, myanesin 150 mg

C Chloralose 0 06 g per kg, strychnine 0 2 mg., myanesin 50 mg

D Chloralose 0 05 g per kg, strychnine 0 4 mg, myanesin 100 mg

The effect of myanesin on the experimentally increased knee jerk was also studied Cats were injected with doses of strychnine insufficient to cause convulsions but producing exaggerated reflex activity. Myanesin in small doses promptly counteracted this increased reflex excitability and caused an immediate reduction of the reflex to the level present before administration of strychnine. (Fig. 3C and D.)

The effects of myanesin on the knee jerk of rabbits anaesthetized with urethane were also examined, they were similar to those observed in cats. Myanesin, 100 mg per rabbit weighing about 3 kg, caused a decrease in the size of a few jerks immediately following the injection. Smaller doses did not influence the size of the jerk but abolished tremors, clonus and spontaneous movements of the leg

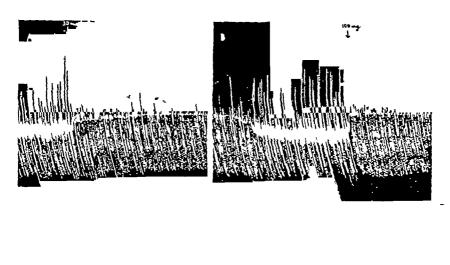


Fig. 4—Record of knee jerk

Cat 3 4 kg

Chloralose 0 08 g per kg

Time interval 30 seconds

At first arrow 30 mg

myanesin, at second arrow 100 mg

myanesin i v

Between A and

B a piece of the record occupying 40 minutes was cut out

The experiments show that although myanesin has little effect on the normal knee jerk of the cat, it is very effective in reducing an exaggerated reflex to its normal size. Myanesin in suitable doses may therefore exert an inhibiting action on pathologically exaggerated functions of the central nervous system without influencing normal reflexes.

DISCUSSION

The experiments reported in this paper show that the effects produced by myanesin are due to its peculiar action on the spinal cord. Myanesin selectively depresses hyperexcitability of spinal reflexes, but hardly influences the normal reflex actions mediated through the cord. Symptoms of hyperactivity, whether due to the action of poisons or to light anaesthesia, can be inhibited with smaller doses of myanesin than those required for the depression of normal physiological functions. Strychnine convulsions in mice can be counteracted with as little as 20 mg per kg intravenously, but 150 mg per kg or more, administered by the same route, are required to paralyse the animal. In cats 20 mg abolished tremors and clonus, but 150 mg did not abolish the knee jerk. The relation between the dose required to bring hyperfunction back to normal and that causing depression of normal function was approximately the same with mice, rabbits, and cats

The depressant action of myanesin on the peripheral nerves (the local anaesthetic action) is too weak to play any part in the effects produced by systemic administration of myanesin. In concentrations which can be achieved after systemic administration, myanesin does not exert any direct relaxing action on

muscles and does not block the action of acetylcholine on them The curare-like action obtained with large doses is of toxicological interest only and never becomes apparent with doses from which animals recover It is incorrect to call myanesin a curarizing agent, because doses causing relaxation during anaesthesia do not influence neuromuscular transmission, but produce relaxation by a depressant action in the spinal cord

Muscular relaxation produced by myanesin differs from that produced by curare not only in that the drugs act on different structures, but also in the order and degree in which various groups of muscles are affected. With curare, muscles with cranial innervation are affected first, the peripheral and intercostal muscles are paralysed next, and with complete curarization the diaphragm is affected to almost the same extent as other muscles. With myanesin the muscles of the posterior half of the body are affected first, next the peripheral and intercostal muscles, then the cranial muscles, the diaphragm is affected last and there is a distinct margin between doses causing muscular paralysis and those causing arrest of respiration

Both curare and myanesin have been used for the production of muscular relaxation during anaesthesia (Mallinson, 1947). Curare is the agent of choice for complete suppression of respiration, such as is required during certain operations on the lungs. Myanesin, on the other hand, appears to be more useful for the production of muscular relaxation when suppression of respiration is not desired. Myanesin should never be used for the production of respiratory arrest, because the doses required for this purpose are large and affect the heart and blood pressure.

Occasionally when an attempt was made to produce respiratory arrest in an already paralysed rabbit by rapid intravenous injection of myanesin, a general rigidity without arrest of respiration developed. The rigidity, which in appearance was similar to decerebrate rigidity, lasted for about 1 min and was followed by profound muscular relaxation from which the animals recovered. The cause of this symptom is not understood. It may be due to a direct action on the muscle. It was observed only in certain rabbits after rapid intravenous injection of concentrated solutions. It was not seen in anaesthetized animals. Other species of animals, as well as most rabbits, showed respiratory arrest if administration was continued after complete paralysis was obtained

The rigidity observed after rapid intravenous injection to certain non-anaesthetized rabbits is the only sign of stimulation which has been observed with myanesin. With this exception myanesin caused pure depression in all species which were examined and at all dose levels. Crystalline d-tubocurarine chloride, on the other hand, may cause conspicuous signs of central stimulation such as trembling, hyperexcitability to stimuli, muscle twitching, and convulsions. These symptoms are particularly well marked in mice and rats, but can also be observed in other species (Cohnberg, 1946)

West (1935) observed that certain samples of curare removed the violent spasm of parathyroid tetany in dogs without paralysing the animal. This selective removal of pathological rigidities without apparent diminution of voluntary power was termed lissive action by West. Pure crystalline tubocurarine chloride possessed no trace of lissive power in dogs.

Experiments reported in this paper indicate that myanesin possesses a lissive action. It should, therefore, prove useful in the treatment of spastic paralysis, hypertonic states, and tremors. It may be expected that myanesin will be effective in these conditions in doses which do not affect consciousness, do not diminish muscular power, and do not cause side effects. Similar results may also be expected after oral administration because myanesin is well absorbed from the intestinal tract.

SUMMARY

- 1 Myanesin in high dilution had no direct action on the rectus abdominis of the frog and did not block the action of acetylcholine on this muscle
- 2 The local action of myanesin on peripheral nerves was similar to that of procaine
- 3 In doses causing reversible paralysis, myanesin did not possess a curarelike action, but toxic doses had a blocking effect on the myoneural junction
- 4 Myanesin had but little effect on the normal knee jerk. An exaggerated knee jerk due to light anaesthesia, strychnine, or unknown causes was promptly reduced to the usual size
- 5 Myanesin in small doses antagonized all the effects of strychnine and relieved tetanic spasm
- 6 Myanesin had a selective depressant action on the spinal cord. In doses which had little effect on voluntary power it restored deranged reciprocal innervation to normal and counteracted symptoms caused by a release from inhibitions as observed during light anaesthesia. The powerful effect of myanesin on tremors, increased reflex excitability, and similar symptoms suggests that it may be useful in the treatment of spastic and hypertonic conditions

I wish to express my thanks to the Directors of The British Drug Houses, Ltd, for their interest in the work and their permission to publish the results. My thanks are also due to Mr R A Hall and to Misses B J O'Brien, L F Carrick, D M Culver, D M Gurney, B Hall E U Haywards and M E L Tattersall for technical assistance

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THE USE OF DRUG ANTAGONISTS FOR THE IDENTIFICATION AND CLASSIFICATION OF DRUGS

BY

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A sensitive biological method for the identification of drugs has been described by Chang and Gaddum (1933) It consists in estimating the activity of an unknown substance in terms of a standard, quantitatively, in several different pharmacological tests. As a rule the ratio of activity between standard and unknown in different tests is only constant if the two samples are chemically identical, otherwise the ratio of activity in different tests varies. By this method even closely related substances may be differentiated

Another biological method for the identification and differentiation of drugs consists in testing quantitatively their responses towards antagonists. It has been shown in a previous communication (Schild, 1947) that the effects of histamine and acetylcholine may be thus distinguished, since many antagonists although unspecific at high concentrations will differentiate between these drugs at lower concentrations. The present investigation is concerned with the question whether drugs which are more closely related than histamine and acetylcholine may also be differentiated in this way, or whether antagonists, even though they may be used in an exact and quantitative manner, would fail to discriminate between closely related substances

If indeed some drugs, although distinguishable chemically or by their relative activity in different pharmacological tests, could not be distinguished by their reactions to antagonists, they might conveniently be grouped into a common pharmacological class. It will be shown that drugs can in fact be found which are related in this way, and that if a certain simple scheme of drug action be accepted a rational classification of active drugs based on their responses to drug antagonists may be attempted

Methods

The experiments were done on the isolated ileum of the guinea-pig, employing the apparatus for assaying antagonists on isolated tissues which has previously been described (Schild, 1947)

Plan of experiments—The object of these experiments was to find out whether drugs could be differentiated by their responses to antagonists. They were accordingly designed to test whether the effects of two given stimulant drugs would be reduced by some antagonist to the same extent or not

The assays are performed in two stages. In a preliminary experiment doses of the two active drugs producing approximately equal submaximal effects are determined as well as a concentration of the antagonistic drug sufficient to reduce these effects without completely abolishing them. In a final experiment these drugs are administered in the presence and in the absence of the antagonist in a planned sequence, which must be statistically unbiased in order to allow a statistical analysis of the results of each experiment to be made

A typical experiment is shown in Fig 1 The order of addition of drugs to the bath is as follows. The doses are administered in sets of four, each set consisting of a random sequence of one dose of each of the active drugs alone and a further dose of each of the active drugs in the presence of the antagonistic drug (after a preliminary period of contact between tissue and antagonist of 2 min.) After each administration of the antagonist several doses of the active drug alone are interjected in order to let the tissue recover, if possible, from the preceding depression

Persistent depression produced by antagonists is the most serious difficulty in planning an unbiased sequence of doses suitable for statistical analysis. The depressant effect is partly overcome by interposing doses of the active drug alone. More fundamentally, the arrangement of the experiment itself is such that each of the active drugs has an equal chance of being preceded by a depressant injection, and it is thus unbiased with regard to the main point at issue. It is merely a matter of convenience how many 'recovery' periods of active drug alone are interposed and it may in some cases be more advantageous to have a fixed small number of such periods rather than to wait until the response has reached a steady state.

A complete experiment consists of several sets (randomized groups) of four doses. The final effect is assessed by averaging the effects obtained in the individual sets. If the two drugs are depressed to the same extent by the antagonist, and \mathcal{F}_A and \mathcal{F}_B are the mean effects produced by drugs A and B, and \mathcal{F}_{AZ} and \mathcal{F}_{BZ} the mean effects produced by these drugs in the presence of the antagonist Z.

$$ar{v}_{A} - ar{v}_{AZ} = ar{y}_{B} - ar{v}_{RZ}$$
 or $ar{v}_{A} + ar{v}_{BZ} = ar{y}_{B} + ar{y}_{AZ}$

The latter equation is tested statistically by an analysis of variance carried out as previously described (Schild, 1942). In practice there is usually some deviation from theoretical equality, but unless this is statistically significant it may be assumed that both drugs have been depressed to the same extent

Use of a Drug Antagonist for the Identification of an Unknown Substance

Curare causes the release of a histamine-like substance from the perfused gastrocnemius muscle of the dog (Alam et al, 1939) In the present experiment a dog's hind limb was perfused with defibrinated blood by means of a Dale-Schuster pump, the blood being oxygenated through the animal's own lungs a solution of 50 mg crude curare was injected into the artery perfusing the limb and the effluent was collected. The blood was extracted by Code's method and the extract assayed on the guinea-pig's ileum. The apparent concentration

of histamine in the sample was 500 μg per 1 as against 16 μg per 1 in the controls

In order to identify the substance further the effect of a low concentration of benadryl (dimethylamineethyl benzhydryl ether hydrochloride) on it was compared with the effect of the same concentration of benadryl on histamine The experiment is illustrated in Fig 1. A concentration of benadryl of 1. 150

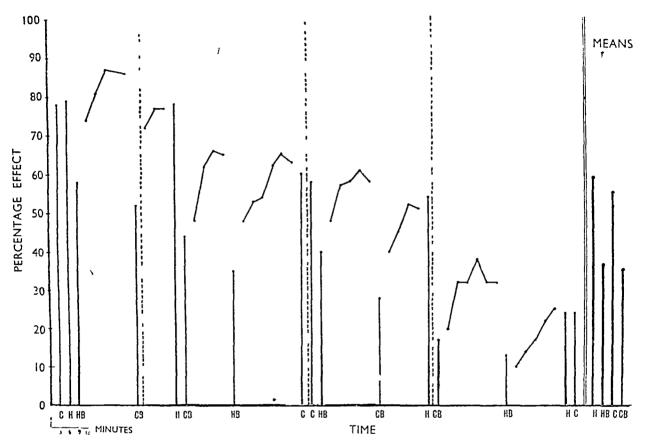


Fig 1—Assay to test whether the effects of histamine (H) and an unknown substance (C) released from skeletal muscle by curare are reduced to the same extent by benadryl (B) Sequence of injections explained in the text. Vertical lines represent the effects of test doses of the two drugs given in the presence and in the absence of the antagonist, each effect being repeated 4 times. The irregular curves represent the effects of a constant dose of histamine given in order to allow the preparation to recover from a preceding dose of benadryl. Note incomplete reversal of depression

million produced an appreciable reduction of the effects of both histamine and the unknown substance. There also occurred a progressive deterioration of the preparation, presumably due to the cumulative effects of the antagonist. In spite of this and the unequal effects produced by individual doses of the antagonist, the mean reduction of effect of the two active drugs is almost identical

The results of an analysis of variance of this experiment are shown in Table I In the last column the mean square for each item should be compared with the mean square for

error Two of the mean squares are smaller than the error mean square and thus statistic ally obviously not significant. One of these is the mean square which assesses the relative reduction of the effects of histamine and the extract by benadry! Since there is no signifi-

TABLE I

ANALYSIS OF VARIANCE OF BENADRYL DEPRESSION OF HISTAMINE AND HISTAMINE-LIKE SUBSTANCE RELEASED BY CURARE

Source of variation	Sum of squares	Degrees of freedom	Mean square
Between successive trandomized groups' Between histamine and extract Between active drugs alone and active drugs + benadryl	4805 25	3 1	1602 25
(slope') Between depression of histamine effect by benadryl and	1764	1	1764
depression of extract effect by benadryl Error	6 502	9	6 56
Total	7102	15	

cant difference the reduction of the two effects may be assumed to be equal and the main object of the experiment is achieved. A further incidental result is a lack of statistically significant difference between the effects produced by histamine and the extract, indicating that the activity of the extract might reasonably be assumed to be 500 µg per l as originally assumed. Two of the mean squares are much higher than the mean square for error and statistically significant. One of these is the mean square for "slope" indicating that the concentration of benadryl used produced a significant reduction of effect. The other is the mean square between successive 'randomized groups," showing that a real change in sensitivity, in this case a progressive deterioration occurred in the course of the experiment.

Similar results were obtained in two further experiments

It was concluded that since benadryl does not discriminate between the two substances they are likely to be related of possibly identical

DRUGS WHICH ARE INDISTINGUISHABLE BY THEIR REACTION TO ANTAGONISTS

has been synthetized by Garforth and Pyman (1935) Its pharmacological actions were investigated by Vartiainen (1935) who found it to be twice as active as histamine on the guinea-pig's ileum and about half as active as histamine on the cat's blood pressure. Since histamine and its N-methyl deriva tive are closely related, as well as clearly distinguishable by their relative activity in different pharmacological tests, they were selected as a representative pair to investigate the effect of antagonists on closely related substances

In the following experiments the effects of three different antagonists on the action of equiactive concentrations of histamine and N-methylhistamine were

studied The N-methyl derivative was approximately 25 times as active as histamine in terms of molar concentrations. The antagonists used were antergan (N N-dimethyl-N'-phenyl-N'-benzylethylene diamine hydrochloride, Halpern, 1942), pethidine, and atropine. The results are illustrated in Fig. 2.

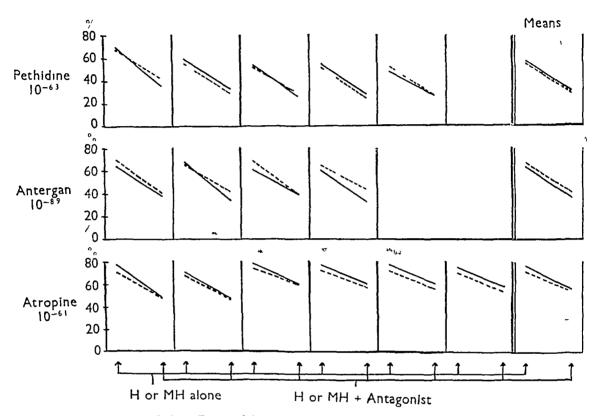


Fig 2—Reduction of the effects of histamine (----) and N-methylhistamine (----) by three different antagonists. The molar concentration of histamine used was approximately 2.5 times that of N-methylhistamine

Points in the top left-hand corner of each square represent the effects of histamine or N-methylhistamine alone, the lower points the effect of these drugs in the presence of a constant concentration of antagonist Successive squares in a horizontal row represent effects in successive randomized groups" and the final square the arithmetical mean of effects, all obtained on the same strip of intestine

The results were analysed statistically as in the previous example and it could be shown that a given concentration of each antagonist produced, within statistical limits, the same reduction of effect of the two stimulant drugs. It was concluded that none of the three antagonists is capable of discriminating between histamine and N-methylhistamine.

These experiments show that certain drugs react in an analogous manner towards antagonists of very different chemical and pharmacological nature. It is doubtful if any antagonist could be found to discriminate between histamine

and its N-methyl derivative If the unknown substance discussed in the preceding section had in fact been N-methylhistamine it could not have been distinguished from histamine by means of these antagonists

Use of Antagonists for the Classification of Drugs

It might reasonably be assumed that drugs which in a given pharmacological test could not be differentiated by their reaction towards antagonists, or, more precisely, drugs which in the presence of any effective antagonist showed equal reductions of equal effects, would be closely related in their pharmacological action on the particular test object used Conversely, drugs which could be differentiated by their reactions towards most antagonists except the most unspecific ones might be said to be pharmacologically unrelated

In thus using the effects of antagonistic drugs as a criterion for classifying active drugs, the following scheme of drug action might perhaps be visualized. It may be assumed that between the first impact of a drug on the tissue and its final effect, muscular contraction in this case, a series of successive processes occur with any of which antagonists can interfere. Two successive stages are indicated in Fig. 3

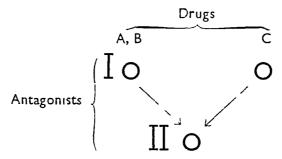


Fig 3—Site of action of antagonists—If A and B stand for histamine and N-methylhistamine and C for acetylcholine, it is possible that small concentrations of antagonists may act at one of the primary sites only, whilst larger concentrations may also antagonize a later common reaction

If two drugs have different primary points of impact their reactions must sooner or later converge into a final common path. An antagonist may interfere with the action of a stimulant drug either before or after a common final path with some other drug is reached. Thus antagonist I reduces only the action of drugs A and B, but not that of C, whilst antagonist II reduces A, B and C. It may further be assumed at least as a first approximation, that an antagonist acting at a given site would reduce the effect of those drugs with whose pathways it interferes to the same extent. Thus antagonist I would depress A and B equally and antagonist II A and B and possibly C equally

It follows that if two drugs such as A and B act by the same mechanism, their effects will be reduced to the same extent by antagonists, independently of whether the latter exert their action by competing for a primary site or by

interfering with some other reaction involved in the contractile process. Such drugs might well be classed together into a primary pharmacological class

Antagonists may of course act at more than one site. A possible explanation for the dual action of drugs such as atropine and neo-antergan (Schild, 1947), which antagonize both histamine and acetylcholine at relatively high concentrations, but only one of the two at low concentrations, would be that at low concentrations the antagonists act at the level of I only and at high concentrations at the level of II as well as of I. Another way of explaining the dual action would be to assume that at high concentrations neo-antergan begins to affect the primary site for acetylcholine and atropine to affect the primary site for histamine. At any rate it is clear that at higher concentrations these antagonists must act on at least two sites.

The scheme is capable of expansion to include the action of further groups of drugs and their antagonists. A scheme of this kind cannot, however, account for special types of antagonism, such as a chemical combination between active drug and antagonist

DISCUSSION

One of the earliest attempts at classifying drugs by their reaction to antagonists was the classification of substances contracting plain muscle into musculotropic and neurotropic according to their reaction to atropine. Various objections have been raised from time to time to this conception

It was pointed out by Magnus (1905) that using the criterion of atropine to localize the site of action of drugs in the intestine implied firstly that atropine had only one site of action in the wall of the intestine and secondly that the effect of atropine could not be reversed by some other drug acting on the same Both these assumptions were unlikely to be true It was indeed shown by Magnus himself that atropine had more than one site of action in the intestine and that it would antagonize at different concentrations drugs belonging to quite different groups such as pilocarpine and barium, and it had been shown by Langley and others that there existed a quantitative antagonism between atropine and pilocarpine This line of criticism does not necessarily invalidate the use of drug antagonists for localizing the site of action of drugs, rather it points to the necessity of refining these methods by using drug antagonists in a more In practice the setting apart of a group of "muscarinic" drugs quantitative way (Dale, 1914), exceptionally sensitive to atropine, has been extremely fruitful, and although the localization implied in the term "neurotropic" cannot be maintained any longer, these drugs must still be regarded as forming a group apart, likely to have a common mechanism of action

From another point of view the old classification has been criticized by Winder *et al* (1946) These workers came to the conclusion that a subdivision into two groups was inadequate, since in addition to the acetylcholine group, a histamine and a barium group of plain muscle stimulants could be clearly dis-

tinguished by their differential reactions towards antagonists. There is, however, no special reason for confining plain muscle stimulant drugs to three types only and it would seem reasonable to look for further types to be differentiated by their reactions towards antagonists. This is precisely what the present classiff cation proposes to do, since drugs which react quantitatively alike to antagonists are placed together, and drugs which can be differentiated by their quantitative response to antagonists are separated

The first distinguishing feature of the proposed classification is that it relies upon a quantitative discrimination, the second that it relies upon the response to several antagonists rather than to a single one, only those drugs are assigned to a primary pharmacological class (in relation to a given tissue) which respond in a quantitatively identical manner to every effective antagonist. This method of classification has the merit of being sharply defined, but it may ultimately prove to have been too rigidly conceived. In a more general way, however, quantitative similarity in behaviour to antagonists is bound to denote some pharmacological relationship between drugs, and the recognition of these relationships may eventually lead to a better understanding of the mechanism of action of drugs

SUMMARY

- 1 A method is described for evaluating statistically whether the effects of two drugs are reduced equally by antagonists
- 2 If equal effects are produced on the guinea-pig's ileum by histamine and N-methylhistamine, they are antagonized to the same extent, quantitatively, by effective concentrations of three different antagonists. Similarly the effects of histamine and a histamine-like substance released from striated muscle by curare are equally depressed by an antagonistic drug. It is concluded that antagonists probably cannot be used to discriminate between closely related drugs.
- 3 A scheme of drug action is proposed which can serve as a basis for a classification of active drugs by means of drug antagonists

I am indebted to Dr A L Morrison of the Roche Research Department for preparing N N-dimethyl-N'-phenyl-N'-benzylethylene-diamine, and to Dr Harold King for a supply of N-methylhistamine

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THE INFLUENCE OF CALCIUM AND POTASSIUM IONS ON THE TOXICITY OF OUABAIN

BY

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(Received April 22 1947)

In the mass of literature dealing with digitalis and Ca and K ions there are no quantitative results showing the influence of varying amounts of these ions on the action of a constant dose of glycoside, nor are there any laboratory observations on the effects of Ca or K on the symptom of digitalis vomiting Accordingly the following studies have been made

Anaesthetized rabbits have been used to determine (i) the lethal dose of ouabain infused intravenously at constant rate in physiological saline, (ii) the lethal dose of the same concentration of ouabain infused together with increasing concentrations of CaCl₂, and (iii) the lethal dose of the same concentration of ouabain infused together with increasing concentrations of KCl

Perfused rabbit hearts have been used to determine the effect of alterations in the amount of (a) CaCl₂ and (b) KCl in the Locke solution on the effect of a fixed concentration of ouabain

Pigeons have been used to determine whether the injection of KCl modified the action of ouabain in causing vomiting

EXPERIMENTAL RESULTS

(a) Anaesthetized rabbits

Method —Thirty-four animals were used. All were given urethane by ear vein till full surgical anaesthesia was reached, the dose necessary being remarkably constant at 155 g (62 ml of 25 per cent (w/v) in distilled water) per kg of rabbit given over 10–15 min Urethane was used in order to avoid ether, which increases the scatter of results of digitalis assay by the cat method (Burn, 1937), the quantity agrees well with the 18 g/kg usually recommended for cats intramuscularly. Cannulae were inserted into the trachea, the left carotid artery for recording the blood pressure by mercury manometer, and the left femoral vein for the infusion of solutions. Artificial respiration was given by a pump as soon as an animal's respiration began to be shallow. Into all animals, except controls, ouabain (20 μ g/ml) was infused in physiological saline at the constant rate of 0.15 ml/min/kg rabbit. This rate of infusion is within the optimum range (Rapson and Underhill, 1935). Observations on the effects of Ca and K on the lethal dose of ouabain were made by adding varying amounts of CaCl₂ or KCl to the infusion, and the end-point of an infusion was taken as the point at which blood pressure suddenly fell to zero without recovery. The animals were thus in the following groups

- (i) Receiving ouabain alone
- (ii) Receiving one on a bain +1, 2 or 3 per cent (w/v) CaCl.

- (iii) Receiving ouabain + 05, 1 or 2 per cent (w/y) KCl
- (iv) Controls receiving 3 per cent CaCl, alone

The control animals needed more than 0.5 g CaCl₂/kg to kill them, in agreement with Nahum and Hoff (1937) who gave 10 per cent CaCl₂ at 2 ml/min. In the present series none of the rabbits receiving ouabain and calcium received more than 0.09 g CaCl/kg Since the effects of KCl on digitalis toxicity were protective, no controls with KCl alone were considered necessary

Results—In order to compare the relative effects of CaCl₂ and KCl on the action of ouabain, concentrations were expressed as molarities, but percentage concentrations (w/v) were also recorded for convenience (Table I) The mean results are represented graphically in Fig. 1 in order to show the difference

TABLE I rabbits Femoral vein infusions of 20 μ g ouabain/ml at 0.15 ml/kg/min

Fluid infused		Individual LD ouabain µg/kg rabbit	
Ouabain and CaCl ₂ Amount of CaCl ₂			
Molarity × 10	Per cent		
2 7 1 8 0 9	3 2 1	30 5, 36 5, 35 7, 58 0, 58 0 60 0, 51 3, 73 5, 49 6, 74 7 75 5, 105, 93 3, 65 8, 56 0	43 7 61 8 79 1
Ouabaın alone		114, 77 7, 81 0, 117, 88 0	95 5
Ouabain and KCl Amount of KCl			
Molanty × 10	Per cent		
0 67 1 3 2 7	0 5 1 2	148, 105, 114, 123 121, 145, 132, 166, 141 171, 151, 156, 130	123 141 152

in slope of the curves relating mean LD ouabain (ordinates) to concentration of CaCl₂ or of KCl (abscissae) in the infusion 'Clearly there is a qualitative difference in effect, increased CaCl₂ causing a linear decrease in LD ouabain, while increase of KCl causes an increase in LD ouabain, there also appears to be a quantitative difference, because the curve for KCl is the steeper. With the two lowest concentrations of potassium used, unit change of concentration had a greater effect on the LD of ouabain than had unit change in concentration of CaCl₂, thus, 1, 2, and 3 per cent CaCl₂ decreased the LD ouabain by 17, 36, and 54 per cent respectively, whereas 0.5, 1, and 2 per cent KCl increased it by 29, 47, and 59 per cent. This suggests that the action of ouabain is influenced more by the absolute concentration of potassium in a perfusion fluid than by the potassium/calcium ratio, at least over a certain range. In order to

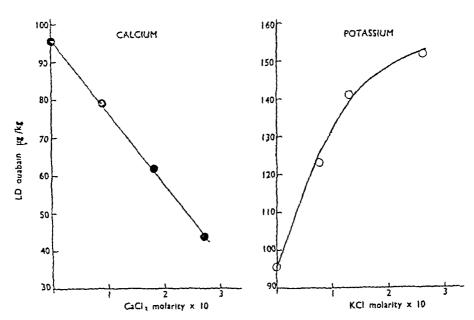


Fig 1—Rabbits Femoral vein infusion of ouabain (20 μg/ml) at a rate of 0.15 ml/kg/min. The relation between the LD ouabain and the Ca or K content of the infusion

test this point it was necessary to perform experiments in which potassium or calcium concentrations could be lowered as well as raised, and accordingly the isolated perfused rabbit heart was used

(b) Perfused rabbit hearts

Method—Thirty-six animals were used. Animals were killed by a blow on the head and bled out by cutting the throat. The heart was rapidly cut out, dissected clean and perfused with oxygenated Locke's solution (percentage composition (w/v) as follows 0.9 NaCl, 0.042 KCl, 0.024 CaCl₂, 0.1 dextrose, 0.05 NaHCO₂) at 36° by the Martin-Langendorff method. A hook was passed through the apex of the ventricles and the amplitude of beat recorded on a smoked drum by a lever and writing point. Rate and amplitude of beat and coronary flow were allowed to become steady, and from 30-45 min after setting up the preparation perfusion was commenced with ouabain (0.4 $\mu g/ml$) in the Locke's solution. The measure of toxicity of the ouabain was taken as the time it took to reduce the amplitude of beat to 50 per cent of its original value. This time is referred to as the "survival time." Thus anything which increased the toxicity of the ouabain shortened the survival time, and anything which decreased the toxicity lengthened the survival time. All hearts except the controls received 0.4 μg ouabain/ml, and were divided into the following groups.

- (1) Receiving ouabain in normal Locke's solution
- (11) Receiving ouabain in Locke with half or twice the normal CaCl.
- (iii) Receiving ouabain in Locke with 05, 125, and 15 times the normal KCl
- (iv) Control hearts receiving no ouabain With the highest and lowest concentrations of CaCl_ or KCl used all hearts survived for long periods
- (v) Hearts, in normal Locke containing $0.4 \mu g$ ouabain/ml, in which toxic effects were caused to disappear by changing to perfusion fluid containing excess KCl

Results —For the reasons already given, the concentrations of CaCl₂ and KCl were expressed in molarities These results (Table II) are plotted graphically (Fig 2) as mean survival times against molar concentrations of CaCl₂ or KCl, and, as for the anaesthetized rabbits, it will be seen that when potassium is increased above normal, unit change in concentration of potassium produces a bigger effect on survival time than unit change in calcium concentration when calcium is decreased. This is expressed more clearly (Fig 3), when the same

TABLE II rabbit hearts perfused by Langendorff's method with 0.4 μg ourbain/ml

Concentration in Locke CaCl ₂ KCl		Ratio	Individual survival times	Mean survival		
Molarity 103	mg per 100 c c	Molarity > 103	mg per 100 c c	K/Ca	in minutes	times in minutes
2 16 4 32 1 08	24 48 12	5 63	42	2 61 1 30 5 22	32, 39, 49, 50, 51, 34 23, 28, 14, 40, 13 60, 39, 45, 28, 49	38 24 44
2 16	24	2 81 7 03 8 44	21 52.5 63	1 30 3 26 3 91	20, 13, 14, 16 92, 110, 45, 48, 66 87, 114, 195, 207, 254	16 72 171

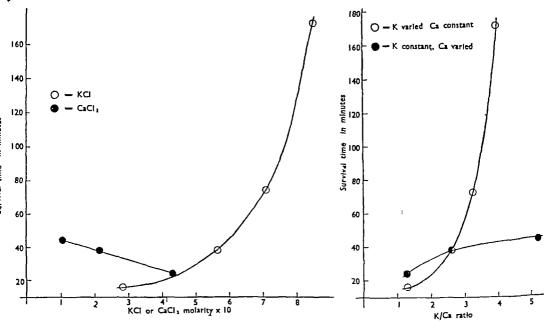


Fig 2—Langendorff rabbit hearts, 04 µg ouabain/ml Locke's solution at 36°C. The relation between the time required to reduce the amplitude of the beat to 50 per cent of its original value ('survival time') and the Ca or K content

Fig 3—Same as Fig 2
The relation between
survival time" and
the K/Ca ratio

survival times are plotted against the K/Ca ratio in the perfusion fluid. If the alteration in the ouabain effect had been solely a function of the K/Ca ratio, the same effect would have been obtained at a given K/Ca ratio whether it was attained by increasing K, or by decreasing Ca, but as Fig. 3 shows the absolute concentration of K had a relatively greater importance

In addition to the quantitative results with potassium, three hearts were perfused with 0.4 μg ouabain/ml in normal Locke until gross irregularities of beat occurred, usually within 25 min, potassium was then added to the perfusion fluid to increase its KCl content by 50 per cent. This abolished the irregular rhythms, caused systole to lessen, and prolonged the survival time, which would have been an hour at most, to several hours. A record of one of these hearts is shown in Fig. 4, and is in agreement with the results of Sampson et al. (1943) on the human being

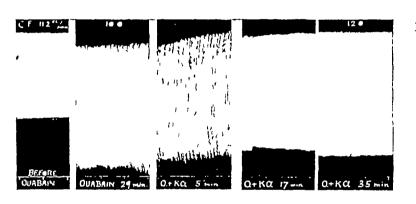


FIG 4—Langendorff rabbit heart KCl abolition of irregular rhythm due to 0.4 µg ouabain/ml Tracing reads from left to right Time marker in minutes Between (i) and (ii) ouabain added to the Locke to contain 0.4 µg/ml Between (ii) and (iii) KCl concentration raised by 50 per cent, with abolition of irregular beats (iv) and (v)

(c) Pigeon emesis

Apparently the effect of K on the vomiting produced by digitalis bodies has never been investigated, except for the observation of Sampson *et al* (1943) that the nausea and vomiting of their patients who received an overdose of digitalis was not affected by potassium acetate administered orally. The pigeon-emesis method of digitalis assay introduced by Hanzlik and Schoemacher (1926), when modified as Burn (1930) suggested, affords a convenient method of testing this action of K against digitalis, if K were to lessen digitalis vomiting, this clearly would indicate antagonism of an extra-cardiac effect of digitalis, since digitalis vomiting has been shown to be independent of cardiac connections with the C N S (Hanzlik and Wood, 1929, Haney and Lindgren, 1942)

Methods—Twenty pigeons were injected with 15 μ g ouabain/300 g pigeon into the wing vein on two separate occasions. The same pigeons were injected on two other occasions (interpolated between these two) with 15 μ g ouabain and 6 mg KCl per 300 g pigeon. This amount of KCl was found to be about the maximum which pigeons would tolerate, for in a trial with KCl alone it killed one bird in a group of 20. The number vomiting is recorded in Table III. When the pigeons were injected with ouabain alone 22 out of 40 injections caused vomiting, with ouabain and KCl, 19 out of 40 injections caused vomiting. Clearly potassium did not protect pigeons against the vomiting induced by the ouabain

TABLE III
PIGEON EMESIS RECORD

Pigeon number	Ouabain 15	μg /300 g	Ouabain 15 μg and KCl 6 mg/300 g		
number	24 3 47	2 4 47	27 3 47	31 3 47	
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20	+++00+++00+000+00+000+0	+++++++++++++++++++++++++++++++++++++++	++0+++00000+000+0000	++++++++++++++++++++++++++++++++++++++	
Total vomiting	10	12	7	12	

DISCUSSION

Although there was reliable information concerning the effects of cardiac glycosides on frog hearts by the middle of the nineteenth century (Vulpian, 1855), it was not until after Ringer's proof of the importance of certain ions in the perfusion fluid (Ringer, 1883) that calcium and potassium became suspected of playing a part in the action of these glycosides In frog hearts either Ca excess or K deficiency increased the action of digitalis, while Ca deficiency or K excess lessened its effect (Werschinin, 1910, Clark, 1912, Konschegg, 1913) From these results it was concluded that digitalis antagonized K just as Ca did Some extreme views emerged, while Burridge (1915-16) postulated that the cardiac glycosides act by sensitizing the heart to calcium, Weizsacker (1917) drew a similar conclusion that digitalis improves the force of the heart only when there is a lack of calcium Loewi (1918) reiterated Burridge's contention, and others wrote in support (Geiger and Jarisch, 1922, Grumach, Grunwald, Handovsky, Hoffmann, Schoen, 1923) Pietrkowski (1918), however, maintained that the effects of low Ca on the action of digitalis could be countered by increasing the sugar of the perfusion fluid, and concluded that digitalis has a direct action on the heart All these observers were concerned with the systolic action of digitalis, and with the systolic effect of calcium, whereas it has been shown (Werschimin, 1910, Cushny, 1925) that small doses of digitalis result in

diastolic arrest Recently this observation has also been brought into line with the theory that digitalis sensitizes the heart to calcium (Blumenfeld and Loewi, 1945)

In mammals the occurrence of calcium and digitalis synergism has been well established both in animal experiments (Lieberman, 1932, Schunterman, 1935, Gold and Kwit, 1937, Gold and Edwards, 1927, Bower and Mengle, 1936, Golden and Brams, 1938), and in the human being, Edens and Huber (1916) finding that patients prone to digitalis pulse bigeminy have a high blood calcium, while Bower and Mengle (1936) record two cases of sudden death after intravenous calcium salts following digitalis

In contrast to the foregoing results are those of Fischer (1928) who found that digitalis sensitizes the heart to all stimuli, e.g., Ca, ethyl alcohol, or K, and of Camp (1939) who also found the heart after treatment with digitalis to be sensitized to K. Nahum and Hoff (1937), however, and Smith, Winkler, and Hoff (1939) failed to obtain Ca and digitalis synergism. Nyiri and DuBois (1930) disagree with the extreme view of Loewi, maintaining that digitalis can exert its full effect in the complete absence of calcium in the fluid perfusing a frog's heart, though they agree that excess of calcium enhances its action

A new line of evidence that K is involved in digitalis action arises first from the work of Calhoun and Harrison (1931) They showed that toxic doses of digitalis lower the level of cardiac K, the effect of therapeutic doses was doubtful Any theory based on these results which suggests that digitalis acts by lowering the K/Ca ratio appears to be untenable, since Calhoun and Harrison also found, in fatal human cases with cardiac failure, that the myocardium of the dilated chambers was low in K Confirmation of this effect of toxic doses has been obtained (Wood and Moe, 1938, Hagen, 1939, Wedd, 1939, Boyer and Poindexter, 1940) though these authors find the effect of therapeutic doses on K content is either negligible or else is to increase it. K loss by digitalis action is also recorded from frog skeletal muscle (Cattell and Goodell, 1937), while further evidence of an effect of digitalis on K metabolism in general is given by Zwemer and Lowenstein (1940), who found that digitalis lowers the plasma K and prolongs life in adrenalectomized animals, thus calling attention to the chemical similarity between the digitalis bodies, especially digitoxigenin, and cortin Dorfman (1940), however, using adrenalectomized mice, was unable to demonstrate any cortin-like activity of strophanthin

Therapeutic advantage has been taken of the antagonism between potassium and digitalis (Sampson, Albertson and Kondo, 1943) in order to alleviate the cardiac effects of overdosage of digitalis by giving oral doses of potassium acetate. No relief of nausea and vomiting was obtained but visual disturbances disappeared

The most recent publications concerning digitalis and heart biochemistry (Chen and Geiling, 1947) show that toxic doses of digitalis diminish cardiac

adenosine triphosphate (ATP), phosphocreatine and adenylic acid, while therapeutic doses have no such effect, and in decompensated hearts the re-synthesis of these three substances was hastened by digitalis administration (Weicker, 1935). These facts, together with the observation that the isolated perfused rabbit heart in systolic contracture from digitalis can be temporarily restored by ATP (Chen and Geiling, 1946), tempt speculation that the K/Ca' effects of the cardiac glycosides may exert an influence on the intricacies of the higher energy-liberating phases of the chemical reactions concerned with muscle contraction

The experiments now described give results which agree with those of the majority, increased calcium potentiates, and increased potassium antagonizes digitalis glycosides. The quantitative aspect takes the matter further, demonstrating the relative importance of the absolute concentration of potassium in the cardiac action of the glycosides, while the failure to protect pigeons against their emetic effect suggests that different biochemical mechanisms are concerned in the cardiac and emetic actions at least

The ability of potassium salts not only to delay the toxic action of digitalis, but also to remove toxic effects when already developed is not generally known Sampson and his colleagues (1943) gave 5–10 g potassium acetate by mouth as a 25 per cent solution to a series of 14 patients in whom digitalis had produced ectopic beats which were recorded by the electrocardiograph. Only one dose was given on any one day. The authors followed the rise in serum potassium and observed the disappearance of the ectopic beats. This occurred in every patient and outlasted the change in serum potassium. The observations described in this paper add support to these findings and suggest that they are due to the interplay of potassium and digitalis in the heart muscle itself.

SUMMARY

- 1 The LD of ouabain by intravenous infusion in physiological saline was determined on 34 rabbits under urethane
- 2 The effect of $CaCl_2$ and of KCl on the LD of ouabain was observed by adding them to the perfusion fluid 1, 2, and 3 per cent solutions of $CaCl_2$ decreased the LD of ouabain by 17, 36, and 54 per cent respectively, 0.5, 1, and 2 per cent solutions of KCl increased the LD by 29, 47, and 59 per cent respectively
- 3 Survival times were observed of 36 Langendorff rabbit hearts perfused at 36° C with Locke's solution containing 0.4 μ g ouabain/ml. The calcium or potassium concentration was varied in different experiments
- 4 Increased calcium or decreased potassium shortened survival time, while decreased calcium or increased K lengthened it
- 5 The effects of altering the potassium were greater than those of corresponding changes in calcium Halving the CaCl₂ prolonged mean survival time by 6 min increasing the KCl by 50 per cent prolonged it by 133 min

6 In a group of 20 pigeons injected via the wing vein with ouabain (15 µg /300 g pigeon) on two occasions, 22 out of 40 injections caused vomiting On two other occasions with the same dose of ouabain plus KCl (6 mg/300 g pigeon), 19 out of 40 injections caused vomiting The difference is not significant

I wish to thank Prof Burn for the guidance and advice he has given throughout

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THE BLOCKING EFFECT OF BIS-TRIETHYL-AMMONIUM SALTS ON TRANSMISSION IN THE PERFUSED SUPERIOR CERVICAL GANGLION OF THE CAT

BY

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The action of the tetra-ethylammonium ion on the circulatory system was described by Burn and Dale (1914) as a paralysing action on sympathetic ganglia resembling that of nicotine, the same type of action was observed in a series of triethylalkylammonium salts by Hunt (1925-6) The action of tetra-ethyl ammonium bromide on the circulatory system as well as on the sympathetic ganglion has recently been analysed by Acheson et al (1946) They concluded that the predominant effect of the tetra-ethylammonium ion was a block of transmission across autonomic ganglia and that this was sufficient to explain the vaso-depressor effect Dr H R Ing suggested to us that it might be worth investigating the effect on ganglionic transmission of bis-triethylammonium salts of the general formula [Et₃N(CH₂), NEt₃]X₃, where X is the anion, such salts might be expected to have a blocking action on transmission in virtue of the triethylammonium groups and their potency might be expected to vary with the length of the polymethylene chain Four bis-triethylammonium salts have been ethylene bis-triethylammonium bromide (BTE2), prepared by Dr H R Ing, and the trimethylene- pentamethylene-, and decamethylene-bis triethylammonium bromides, denoted by BTE3, BTE5, and BTE10 respectively. which were prepared by Mr R B Barlow

METHOD

Cats were anaesthetized with pentobarbitone and the superior cervical ganglion was prepared by Kibjakow's method (1933) modified by Feldberg and Gaddum (1934). Warm oxygenated Locke's solution was perfused through a cannula in the carotid artery at a pressure of about 120 mm of mercury and the venous outflow from the ganglion was collected. The pre-ganglionic fibres to the superior cervical ganglion were stimulated maximally at a rate of 8 stimuli per second, for a period of 15 sec. An interval of 3 min was allowed between each stimulation. The contraction of the nictitating membrane was

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recorded on a smoked drum by an isotonic lever. The activity of the four bis-triethyl-ammonium compounds was compared with that of tetra-ethylammonium bromide (TE) Each drug was given in 0.1–0.2 ml Locke's solution and was injected into the arterial cannula 1 min before the stimulation. A cumulative effect by the drug is very liable to occur when the perfusion rate becomes slow, it was therefore decided to test the drugs in one order and then in the reverse order



Fig 1—Comparison of blocking effect of tetra-ethylammonium bromide (TE) and of two bis-triethylammonium bromides (BTE2 and BTE3) on transmission in sympathetic ganglia Perfused superior cervical ganglion of the cat. Record of the contractions of the nictitating membrane to maximal preganglionic stimulation Rate of stimulation 8 per sec for 15 sec every minute Figures above each contraction=outflow in drops per minute

RESULTS

Fig 1 shows the effect of BTE2 and BTE3 in comparison with that of TE on the response to preganglionic stimulation in the same preparation. The figures above each contraction of the nictitating membrane represent the venous outflow in drops per minute.

It was found that the blocking effect of the five compounds examined was in the order

The relative potency of these five compounds (according to their weight) has been determined on seven preparations, and, giving TE a value of 100, the mean

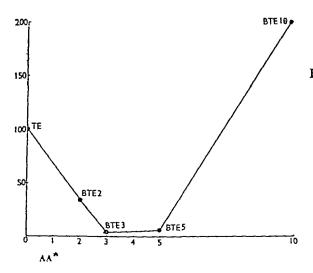


Fig 2—The relationship between the activity of tetra-ethylammonium bromide (TE), given a value of 100, and of bis-triethylammonium bromides (BTE2, BTE3, BTE5, and BTE10) in the perfused sympathetic ganglion of the cat Ordinate percentage potency Abscissae Number of carbon atoms in the polymethylene chain of the molecule

figures are expressed in brackets. No excitatory action on the ganglion was observed with TE, nor with the four bis-triethylammonium compounds

 F_{1g} 2 shows the relationship between the activity of these compounds and the number of carbon atoms in the polymethylene chain of the molecule

DISCUSSION

In their two successive papers, Acheson et al (1946) concluded that the tetra-ethylammonium ion exerts a purely blocking effect on ganglionic transmission. Unlike tubocurarine, the tetra-ethylammonium ion on injection never causes a contraction of the nictitating membrane, nor does it increase the response to electrical stimulation, as intocostrin was shown by Acheson et al to do. It has only a "nicotine-like paralysing" action on the autonomic ganglion. The bistriethylammonium compounds also showed no excitatory action. No experiments have been made in order to discover whether they have any muscarine actions. In general, the action of these compounds is very similar to that of tetra-ethylammonium salts. As shown in Fig. 2, lengthening of the carbon chain between the two onium ions leads to a decrease in activity until the three carbon chain compound (BTE3) is reached, with a further lengthening of the carbon chain there occurs an increase of the blocking effect, and the 10 carbon chain compound (BTE10) is twice as strong as TE

SUMMARY

Four bis-triethylammonium compounds have been tested on the perfused superior cervical ganglion of the cat. They have no stimulating action, but they paralyse ganglionic transmission. There is a relationship between the relative activity of these compounds and the number of carbon atoms in the polymethylene chain of their molecules. The decamethylene-bis-triethyl-ammonium bromide (BTE10) was found to be the most potent compound

We wish to thank Prof J H Burn and Dr Edith Bülbring for their guidance and encouragement in this work

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THE PERSISTENCE IN THE BLOOD STREAM OF SOME ANALOGUES OF SULPHADIMETHOXYPYRIMIDINE

BY

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An account has already been given of the properties of sulphadimethoxy-pyrimidine (I), which displays marked persistence in the blood stream after oral or parenteral administration (Gage et al., 1947)

$$H_{2}N \longrightarrow SO_{2}NH < N \longrightarrow OCH_{3}$$

The availability of some of its homologues (Rose and Tuey, 1946) prompted a comparative examination of their persistence, further compounds were specially prepared by Drs F L Rose, E H Hoggarth, and E H P Young Twenty compounds in all were examined in mice for absorption and persistence The majority were also tested as antibacterial agents, an account of their tuberculostatic activity in vitro and in vivo will be published separately (Hoggarth, Young, and Martin, 1948) No compound appeared likely to have marked therapeutic value. Three (III, VII, and XVI), as well as sulphadimethoxypyrimidine itself, had previously been examined by van Dyke et al (1945), their findings in general parallel those described here

EXPERIMENTAL SECTION

The standard techniques used have been described in previous publications (Rose and Spinks, 1946, 1947, Gage et al, 1947) Each compound was administered orally to a group of three mice as a 1 per cent (w/v) solution of the sodium salt, or as a 1 per cent (w/v) dispersion, in doses of 250 mg/kg. It was then estimated in pooled tail blood, at standard intervals after dosing, by the micro-method of Rose and Bevan (1944). At least six groups of three mice were used for each compound (except XXI). No statistical comparison was attempted, because many compounds were so highly persistent that the maximum concentration was difficult to determine accurately in individual experiments. Values of maximum

concentration (Max), the time after dosing at which this was attained (t max), and the persistence in the blood (expressed as the time (C7) taken for the concentration at 7 hours to fall to two-thirds of that value), were read from the mean blood concentration-time curves

TABLE	•
COMPARISON OF ANALOGUES OF SULPHADIMETHO	XYPYRIMIDINE

Group	Compound	Max blood	concentration	C7	Number	
Group	No	mg /100 ml Time (min)		(hours)	of mice	
A	I	14 4	210	16 5	66	
	II	13.8	100	15 3	18	
	III	11 8	90	13 0	18	
	IV	5 8	540 (?)	>17 0	24	
	V	2 2	180	7 8	21	
В	VI	5 7	180	10 4	21	
	VII	2 1	210	17 0	18	
С	VIII	4 6	150	11 0	39	
	IX	2 3	- 210	10 5	18	
	X	5 7	- 270	9 0	30	
D	XI	12 6	90	47	18	
E	XII	24 7	-90	7 7	30	
	XIII	5 7	120	8 6	27	
	XIV	5 9	40	6 0	18	
F	XV	13 8	60	7 5	18	
	XVI	18 6	65	5 5	18	
	XVII	13 7	45	7 6	18	
G	XVIII XIX XX XX XXI	7 0 3 5 0 9 5 1	40 40 40 150	7 1 * * †	18 18 21 6	

^{*} Disappears very rapidly from the blood † Disappears rapidly from the blood

RESULTS AND DISCUSSION

Compounds have been classified on the basis of their chemical structure into seven groups, each of which is considered separately, the characteristic values obtained from the mean concentration-time curves are given in the Table

Group A 2-Sulphanilamido-4 6-di-n-alkoxypyrimidines

The mean concentrations of these compounds (except II) found in the blood of mice at intervals after the oral administration of 250 mg/kg, are recorded in Fig 1, the characteristic values obtained from the mean curves are compared in the Table Compounds I–IV are highly persistent, the di-n-butoxy homologue (V) is fairly persistent. The other main difference between the five compounds is in maximum blood concentration, which falls with increasing molecular weight, this effect can probably be ascribed mainly to reduced solu-

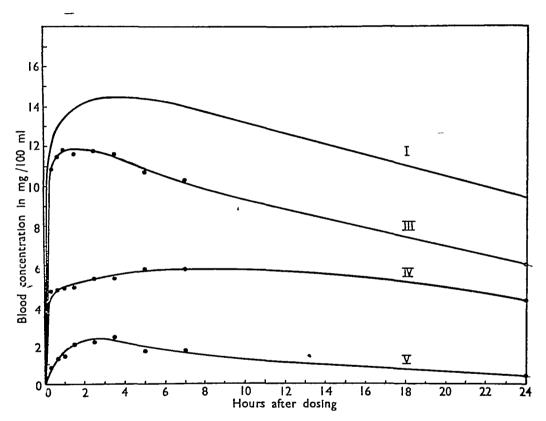


Fig 1—Blood concentrations in mice of sulphadimethoxypyrimidine (I), sulphadiethoxypyrimidine (III), sulphadien-propoxypyrimidine (IV) and sulphadien-butoxypyrimidine (V)

bility of the higher homologues, which would result in a decreased concentration gradient between lumen and blood stream, and therefore in decreased speed and extent of absorption. The solubility data reported by van Dyke et al. (1945) for I and III, and an extensive series of related compounds, support this view. The practical importance of the effect is pointed out by Hoggarth et al. (1948), who show that in vitro tuberculostatic activity increases with increasing molecular weight. It is probably the accompanying decrease in blood concentration which prevents the higher homologues from showing marked therapeutic action in vivo

Group B 2-Sulphanilamido-5-alkyl-4 6-dimethoxypyi imidines

These two compounds are both highly persistent (see Table), but attain lower maximum blood concentrations than the parent compound. The reduction in maximum concentration is greater than when the additional carbon atoms are substituted in the alkoxy group—ie, VI and VII give lower concentrations than their isomers II and III. This was unexpected, since in other groups of compounds it has been found that the distribution of a given number of methylene groups between three, instead of two, normal alkyl radicals leads to increased blood concentrations. The effect may be due to the exceptionally low solubility of this type (van Dyke et al, 1945, Rose and Tuey, 1946)

Group C Branched chain sulphadialkoxy derivatives

OR

$$H_2N$$
 SO_2NH
 N
 OR

Compound No
 $VIII$
 IX
 X
 R
 $ISO-C_3H_7$
 $ISO-C_4H_9$
 $Sec-C_4H_9$

The characteristic values of the three compounds are summarized in the Table They are all highly persistent, the butoxy compounds somewhat less so than the isopropoxy compound (cf Group A) VIII and IX resemble their normal isomers (IV and V) in maximum blood concentration, X gives a much higher maximum concentration than its isomers V and IX. In some other homologous series, e.g., of sulphones, it has been observed that compounds containing branched alkyl chains give much higher blood concentrations than their isomers containing straight chains. It is hoped to describe examples in future publications

Group D 2-Sulphanilamido-4 6-diethoxyethoxypyrimidine

The ethoxyethoxy compound (see Table) has lost much of the persistence characteristic of the other dialkoxypyrimidines, and gives much higher blood

concentrations It should, of course, be compared with its closest analogue the di-n-butoxy derivative (V) The methoxyethoxy homologue was examined by van Dyke et al. They found that it was well absorbed, but of low persistence. Its solubility (4 mM/l at pH 65 and 37°C) was five times that of the dimethoxy compound (0 8 mM/l), and about thirty times that of the diethoxy compound (0 12 mM/l). It may be assumed that the facile absorption of these alkoxyalkoxy compounds is related to their relatively high solubility, but a different effect must cause the low persistence. The relatively low persistence of the di-n-butoxy compound (Group A) suggests that this effect might be connected with steric hindrance, possibly, the bulky alkoxy groups of these compounds hinder their access to some other molecule with which they must be associated to exhibit marked persistence

Group E 2-Metanilaniido-4 6-dialkoxypyrimidines

OR

OR

NH₂

SO₂NH

NH₂

OR

Compound No

XII XIII XIV

R

$$CH_3$$
 n - C_3H_7
 t so- C_3H_7

The metanilamides are compared in the Table Reference should also be made to the properties of the corresponding para isomers. Each metanilamide differs from its para isomer in giving higher maximum blood concentrations, and in disappearing more rapidly from the blood stream. Nevertheless, all three compounds are fairly persistent, approximately of the same order as sulphamerazine (Rose and Spinks, 1946)

Group F 2-Sulphanilamido-4-alkoxypyrimidines

$$R_1$$
 $Compound\ No\ XV\ XVI\ XVII$
 R_1
 CH_3
 The results with these three compounds are given in the Table They are all fairly persistent, approximately of the same order as the metanilamides (Group E) and sulphamerazine, but less so than the dialkoxy derivatives The mean curve for XVII is given in Fig 2

Group G Miscellaneous compounds

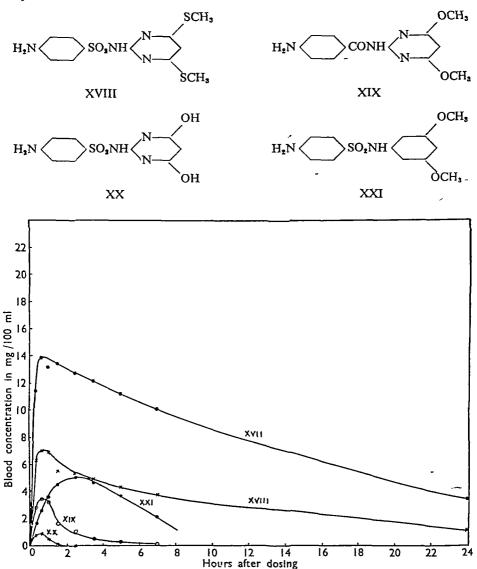


Fig 2—Blood concentrations in mice of sulphamethylisopropoxypyrimidine (XVII), sulphadimethylmercaptopyrimidine (XVIII), 2-p-aminobenzamido-4 6-dimethoxypyrimidine (XIX), sulphadihydroxypyrimidine (XX), and 3 5-dimethoxy-1-sulphanilanilide (XXI)

These compounds show striking differences from those previously described (Fig 2, Table) The dimethylmercapto analogue of sulphadimethoxypyrimidine is less persistent than the latter, and gives a much lower maximum concentration. The other three compounds disappear very rapidly from the blood stream. The low concentrations they attain may be associated with this rapid disappearance.

from the blood rather than with poor absorption. They clearly show no resemblance whatsoever to sulphadimethoxypyrimidine, although each retains certain structural features of the latter

Considering the results as a whole, it is clear that marked persistence in the blood stream is conferred by the presence in the sulphapyrimidine molecule of two alkoxy groups in positions 4 and 6 of the pyrimidine ring. All modifications of this structure result in reduction of persistence, including removal of one alkoxy group, transfer of the *para* amino group to the *meta* position, substitution of a benzene ring for the pyrimidine ring, or substitution of a carboxamide group for the sulphonamide group. The persistence of the dialkoxy compounds appears to fall with increasing molecular weight, although one compound (IV) is a marked exception to this rule

Although the precise nature of the physical and physiological factors which confer persistence on a compound is at present unknown it seems probable that they include a high degree of binding to the plasma proteins and a high degree of tubular reabsorption (Fisher et al, 1943, Beyer et al, 1944, Earle, 1944, van Dyke et al, 1945) The strength of the bond uniting drug to protein may also be of importance (Gregerson and Rawson, 1943, Rawson, 1943) dimethoxypyrimidine and some related compounds have been shown to be extensively bound to plasma proteins (van Dyke et al, 1945, Gage et al, 1947), but no information is available on the other important factors. A high degree of protein binding alone would not necessarily result in high persistence, absorption by the tubules being of equal or greater importance (Fisher et al, 1943, Besides these factors, which influence the rate of excretion Lundquist, 1945) of a compound by the kidney, and others, less adequately investigated, which influence the rate of excretion into the intestine (cf. Silber and Clark, 1946), diazotizable amines are also removed from the blood stream by conversion to non-diazotizable or rapidly excreted metabolites, such as acetyl derivatives, sulphates or glucuronides It is improbable that the persistence, even of closely related compounds, is uniformly affected by such metabolic processes

The complexity of all these mechanisms, which influence persistence, is such that speculation on the nature of the correlation between structure and persistence is hardly justifiable. However, it is clear that the correlation is a very delicate one. It had been hoped that 4 6-dimethoxypyrimidine, or even *m*-dimethoxybenzene, might behave as a "conductophoric" group, and confer persistence on any molecule containing it, in the same manner as the dialkylamino-alkylamino chain of mepacrine, pamaquin, and 3349 (Magidson *et al.*, 1934, 1936, Spinks and Tottey, 1946), or the biguanide chain of paludrine and related drugs (Spinks, 1946, 1947), has been presumed to confer favourable pharmacological properties on the antimalarial containing it. The reduced persistence of compounds XII, XIII, XIV, XIX, and XXI strongly suggests that the introduction of 1 3-dimethoxy groups into a nucleus other than pyrimidine, or the combination of

4 6-dimethoxypyrimidine as a "conductophoric" group with a "toxicophoric" group other than sulphanilamide would be unlikely to confer high persistence on the resulting molecule

SUMMARY

The absorption and persistence in mice of twenty compounds related to sulphadimethoxypyrimidine have been described. High persistence is a property of 2-sulphanilamidopyrimidines carrying dialkoxy groups in positions 4 and 6. Removal of one alkoxy group, transfer of the p-amino group to the meta position, substitution of a benzene ring for the pyrimidine ring, or substitution of a carboxamide group for the sulphonamide group, results in reduced persistence. In each homologous series examined, maximum blood concentration fell, with increasing molecular weight.

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THE GLUCOSE METABOLISM OF *PLASMODIUM GALLINACEUM*, AND THE ACTION OF ANTIMALARIAL AGENTS

BY

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(Received May 1 1947)

Studies on the metabolism of the erythrocytic forms of plasmodia were initiated by Christophers and Fulton (1938), who showed that monkey red cells infected with P knowless consumed oxygen independently of glucose, though added glucose was utilized, the oxygen uptake of the parasitized cells was completely inhibited by cyanide and Coggeshall (1941) showed that parasitized red cells were dependent to some extent on glucose, and that mannose, fructose, and glycerol would replace glucose as a metabolite During the war years, a lot of research on the metabolism of the malaria parasite has been done by various groups This work has been of workers in America comprehensively reviewed in Federation Proceedings (vol 5, No 3) It was reported that, under aerobic conditions, red cells infected with malaria parasites could metabolize lactate, pyruvate, succinate, fumarate, and amino-acids, besides glucose, and that glucose was converted to lactic acid by phosphorylation, as in muscle (Evans, Ceithaml, Speck, and Moulder, 1945, Bovarnick, Lindsay, and Hellerman, 1946a, b, McKee, Ormsbee, Aufinsen, Geiman, and Ball, 1946) Speck and Evans (1945a) showed that cell-free extracts of plasmodia would phosphorylate glucose similarly to veast extracts

Regarding the action of antimalarials, Silverman, Ceithaml, Taliaferro, and Evans (1944) reported that quinine and mepacrine (atebrin) inhibited the respiration of p'asmodia, though the inhibition was delayed until the third or fourth hour of incubation with the drug Speck and Evans (1945b) reported that quinine and mepacrine inhibited the phosphorylation of glucose by cell-free extracts of plasmodia and of normal red cells, and that the degree of inhibition corresponded to the amount of hexokinase activity present. Investigating isolated enzyme systems, these workers

showed that quinine and mepacrine inhibited yeast hexokinase, but not 3-phosphoglyceraldehyde dehydrogenase Lactic dehydrogenase from plasmodium extracts and from ox-heart was inhibited more by mepacrine than by quinine More recent work by Bovarnick, Lindsay, and Hellerman showed that separated thoroughly exhausted of substrates by washing and preliminary incubation, were more sensitive to antimalarial drugs The recovery of respiration after addition of glucose to the exhausted parasites was strongly inhibited by mepacrine, but no inhibition was observed with other substrates hibition of glycolysis by mepacrine was antagonized by adenylic acid or adenosine triphosphate

The present paper, reporting preliminary work on the metabolism of plasmodia, confirms generally the above reports on the glucose metabolism of malaria parasites, and presents fresh evidence of glycolysis by phosphorylation and on the mode of action of quinine and mepacrine

MATERIALS AND METHODS

Blood from chicks 2-4 weeks old, heavily infected (70-95 per cent of cells parasitized) with P gallinaceum, was used as the source of parasite material Since, in initial experiments, whole blood suspensions showed no response to added glucose, washed, parasitized red cells were used in most of the experiments The chicks were killed by placing in an atmosphere of CO2, and blood drawn from the exposed heart was suspended in isotonic buffer (0 85 per cent NaCl, 100 ml., M/15 phosphate buffer, pH 73, 30 ml) containing a little citrate to prevent coagula-The suspension was centrifuged and the clear supernatant discarded, the red cell laver was shaken with more isotonic buffer, re-centrifuged, and the red cells finally suspended in sufficient isotonic buffer to produce the required volume of suspension treatment removed all but a trace of indigenous glucose from the red cells

Respiration experiments—Oxygen consumption was measured in conventional Warburg respirometers, using 15 ml flasks, maintained at 38° C. Determinations were carried out with a set of twelve respirometers, in duplicate, triplicate, or quadruplicate groups, according to the number of variants required. The amount of oxygen consumed was expressed in microlitres (µl) per 1,000 million red blood cells, the percentage of cells parasitized being 70-95

Glucose was determined in the incubates from the respirometer flasks by the method of Folin and Wu (1920) Pyruvic acid was determined by the modified method of Lu (1939) described by Umbreit, Burris, and Stauffer (1945), and lactic acid by the method of Barker and Summerson (1941)

Experiments on glucose phosphorylation—Aliquots of a suspension of washed, parasitized red cells were incubated at 41° C with a known amount of glucose, in contact with various inhibitors and antimalarial agents. A control aliquot was deproteinized immediately by adding an equal volume of ice-cold 12 per cent (w/v) trichloroacetic acid. The test aliquots were incubated for 4-5 hours, during which they were oxygenated either by continuous bubbling, or at frequent intervals. At the end of the incubation period, the test aliquots were deproteinized, chilled in the cold-

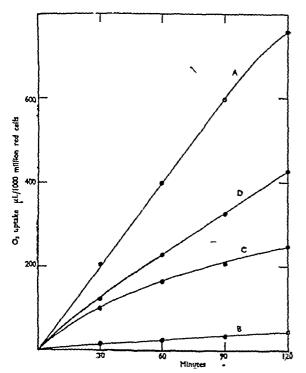


Fig 1—Oxygen uptake of normal and parasitized chick blood (single experiment) A—Whole blood suspension (parasitized) B—Whole blood suspension (normal) C.—Washed, parasitized red cells D—Washed, parasitized red cells + glucose (0 5 mg./ml)

room, and the precipitated protein removed by centri fuging

The clear supernatants were subjected to a complete analysis for glucose, phosphorylated intermediates, and pyruvic and lactic acids. Glucose was determined in dilutions of the supernatants by the method of Folin and Malmros (1929), and pyruvic and lactic acids by the methods already referred to The remaining supernatant—was adjusted to pH 8.2 and analysed for hexose phosphates, triose phosphates, and phosphoglyceric acid, using the experimental technique described by Umbreit, Burris, and Stauffer (1945). The amounts of the various intermediates were expressed as gram-molecules.

> RESULTS

Respiration experiments

Though normal chick red cells showed a sub stantial oxygen uptake (compared with non nucleated mammalian red cells), the respiration rate of heavily parasitized (96 per cent) chick red cells was about 15 times as rapid (Fig 1) The respiratory rate of infected red cells (whole blood suspensions) increased proportionately with the number of cells parasitized (Table I) The oxygen

TABLE-I
THE RELATION BETWEEN OXYGEN UPTAKE AND THE
NUMBER OF RED CELLS PARASITIZED WITH P
gallinaceum

Whole blood suspensions, no added substrate

% cells parasitiz e d	No of respirometers	Ο, uptake (μl/hr/1000 million red cells)
normal "" "" "" "" "" "" "" ""	6 6 0 5 7 2 2	65 4 48 4 27 5 36 2 25 5 19 5 17 1
30 30 44 80 85 88 90	5 5 3 5 5 2 8	77 8 89 0 146 0 250 0 296 0 235 0 314 0

uptake of infected whole blood suspensions was not increased, but actually depressed by adding glucose. If, however, the parasitized red cells were washed free from indigenous glucose, the oxygen uptake was reduced to 37 per cent (mean of 9 determinations, range 27 8-50 8 per cent) of that of the whole blood suspension (Fig. 1). The respiration of glucose-free infected cells was stimulated by added glucose, the recovery being graded

TABLE II

EFFECT OF GRADED CONCENTRATIONS OF GLUCOSE
ON THE OXYGEN UPTAKE OF WASHED, PARASITIZED
RED CELLS

Glucose	% of O ₁ uptake	% recovery of
added	of whole	lost
(mg /3 ml)	blood suspension	respiration
0 05	43 7	6 2
0 1	38 0	nıl
05	57 2	28 7
05	56 5	35 5
05	52 1	26 3
		Mean 30 2
1 0	60 7	42 0
1 0	65 4	36 5
1 0	65 4	42 4
1 0	66 3	51 5
~		Mean 43 1
$\begin{smallmatrix}2&0\\2&0\end{smallmatrix}$	81 7 73 0	71 8 45 1
		Mean 58 5
5 0	84 0	75 4
10 0	80 4	70 0

up to 5 mg/3 ml, when a maximum recovery of 75 per cent of the lost respiration rate was attained (Table II)

The quantity of oxygen consumed by plasmod₁a was not accounted for by the amount of glucose used, for example, when infected red cells were washed, the oxygen consumption was still about 40 per cent of that of the whole blood suspension, though the amount of available glucose was ve₁y

TABLE III
QUANTITATIVE UTILIZATION OF GLUCOSE BY PLASMODIA

(a) Effect of graded concentrations on whole blood suspension

(b) Effect of washing the infected red cells

Preparation	Conc of glucese g /100 ml	Mols O ₁ used per mol glucose consumed	mols lactic acid formed per mol glucose consumed
(a) Whole blood suspension	added nil* 0 01* 0 05* 0 10*	53 31 21 19	4 0 2 5 1 56 1 95
(b) Whole blood suspension Washed cell	present 0 002	4 04	_
suspension	0 00005	26 0	

^{• + 0 02} g /100 ml indigenous glucose

small Thus the oxygen consumption per molecule of glucose used was apparently increased (Table III) Again, since additional glucose did not increase the oxygen uptake of infected whole blood suspensions, the oxygen consumption and the amount of lactic acid formed per molecule of glucose appeared to decrease with increasing concentration of glucose (Table III) No pyruvic acid

TABLE IV

QUANTITATIVE UTILIZATION OF GLUCOSE BY WASHED,

PARASITIZED RED CELLS

Time after adding glucose (min)	μ-mols glucose used	μ mols extra O ₁ used	Mols O ₂ used per mol glucose consumed
30	1 15	0 69	0 60
60	1 70	1 32	0 78
90	2 73	2 68	0 98
120	2 76	3 08	1 12

was formed Table IV shows the course of quantitative utilization of glucose added to washed, parasitized red cells, and the amount of *extra* oxygen consumed Neither lactic nor pyruvic acids accumulated in this instance

Substrates metabolized by plasmodium—The oxygen uptake of washed, parasitized red cells was stimulated by glycerol, pyruvate, and lactate to the same extent as by glucose, but was only slightly stimulated by succinate and fumarate Glutamate, aspartate, and tyrosine were inactive (Table V)

TABLE V

EFFECT OF ADDED SUBSTRATES ON THE OXYGEN UPTAKE OF WASHED, PARASITIZED RED CELLS

Substrate added	Concentration (mg/3 ml)	% stimulation of oxygen uptake
Glucose Tyrosine	1 0 1 0	75 6 nıl
Glucose Pyruvate Lactate Succinate	2 0 2 0 2 0 2 0 2 0	155 161 179 13 5
Fumarate Glucose Gly cerol Glutamate Aspartate	2 0 2 0 2 0 2 0 2 0 2 0	12 9 174 207 nil nil

Effect of specific inhibitors—Cyanide strongly inhibited the oxidation of glucose, lactate, and pyruvate by washed, parasitized red cells Iodoacetic acid, in low concentration (1 in 30,000),

strongly inhibited glucose, but not pyruvate, oxidation, at higher concentration (1 in 6,000), the specificity of inhibition disappeared, and both substrates were equally inhibited (Table VI)

TABLE VI

EFFECT OF SPECIFIC INHIBITORS ON THE OXYGEN
UPTAKE OF WASHED, PARASITIZED RED CELLS ALONE,
AND WITH ADDED SUBSTRATES

Inhibitor	Conc	Percentage inhibition of O, uptake					
Indibitor	(mg / 3 ml)	No added substrate	Glucose	Pyruvic acid	Lactic acid		
NaCN Iodoacetic	0 5	80 4	87 5	93 7	87 7		
acid	0 5 0 5 0 1	36 0	42 0 76 0 63 5	86 0 83 6 10 1	74 6		

Effect of antimalarial agents—Quinine and mepacrine did not markedly inhibit the oxygen uptake of plasmodia either when respiring alone (washed, parasitized red cells), or in the presence of active substrates (Table VII) At fairly high concentration (1 in 6,000), there was little differentiation between the substrates, but at lower concentration (1 in 60,000), quinine showed a slightly greater inhibition of glucose and lactate oxidation, and mepacrine of lactate oxidation (Table VII)

TABLE VII

EFFECT OF QUININE AND MEPACRINE ON THE OXYGEN

UPTAKE OF WASHED, PARASTTIZED RED CELLS ALONE,

_AND WITH ADDED SUBSTRATES

Antı	Conc	Percentag	Percentage inhibition of O, uptal after 2 hours				
malarial agent	(mg / 3 ml)	No added substrate	Glucose	Pyruvic acid	Lactic acid		
Quinine	0 5	22 4	25 7	29 1	35 7		
	0 05	6 0	17 3	2 3	18 9		
Mepacrine	0 5	11 9	34 4	25 6	34 5		
	0 05	9 0	2 0	5 8	14 6		

At still lower concentration (1 in 300,000), the inhibitory effects, particularly of mepacrine, did not appear until the drugs had been incubated with the parasites for 2-6 hours (Table VIII)

Glucose phosphorylation

Changes in glucose, phosphorylated intermediates, and lactic and pyruvic acids during incubation of infected red cell suspensions were expressed as molecules per 100 molecules of glucose used by uninhibited, parasitized red cells

Table IX shows the comparison between the glu cose metabolism of uninfected chick red cells and parasitized red cells. Parasitized cells used about four times as much glucose as normal red cells, and, with parasitized cells, a large amount of lactic acid accumulated (0.5 to 1.5 molecule per molecule of glucose used). Glucose-6-phosphate, fructose-6-phosphate and fructose-1, 6-diphosphate were metabolized more rapidly in parasitized cells than in normal cells, but triose phosphate and pyruvic acid accumulated to a greater extent than in un infected red cells. These observations showed that active glucose phosphorylation was proceeding more rapidly in infected red cell incubates than in normal cell incubates.

TABLE VIII
INHIBITION OF OXYGEN UPTAKE OF WASHED, PARASITIZED RED CELLS BY LOW CONCENTRATIONS OF
OUINING AND MEPACRINE

Antı malarıal	Substrate	Percentage inhibition aft			
agent		2 hr	4 hr	6 hr	
Quinme (0 01 mg / 3 ml)	none glucose	nil 94	5 4 15 7	16 8 20 0	
Mepacrine (0 01 mg / 3 ml)	none glucose	nil nil	nıl 6 8	3 I 9 8	

Effect of specific inhibitors—Iodoacetic acid completely inhibited the utilization of glucose and accumulation of lactic acid, and, in the accumulation of hexose and triose phosphates, showed the typical effects of phosphoglyceraldehyde dehydro genase blockage. Cyanide inhibited the utilization of glucose by 40 per cent, but the amount of lactic acid formed was greater than in the controls. Cyanide stimulated the utilization of phosphory lated intermediates and of pyruvic acid, as shown by a decrease during the incubation period.

Effect of antimalarial agents—Quin ne inhibited glucose utilization more powerfully than mepacrine did. The most marked effect of mepacrine was the large accumulation of adenosine triphosphate (ATP), indicating inhibition of the enzyme hexokinase by which glucose is initially phosphorylated Quinine also inhibited hexokinase, but to a smaller extent than mepacrine Both drugs caused accumulation of glucose-6-phosphate, but not to the same extent as iodoacetic acid. In the presence of quinine, pyruvic acid accumulated, mepacrine reduced the accumulation of lactic acid more than quinine did

TABLE IX

EFFECT OF SPECIFIC INHIBITORS AND ANTIMALARIALS ON GLUCOSE PHOSPHORYLATION BY WASHED CHICK RED CELLS INFECTED WITH P gallinaceum

Figures denote the mean numbers of μ mols of the substrates formed (+) or used (-) per hour per 100 μ mols of glucose consumed by the control suspension

No of expts	Inhibitor	Molar conc of inhibitor	Glucose	АТР	Glucose-1- phosphate	Glucose-6-	Fructose-6- phosphate	Fructose-1, 6- diphosphate	Trose phosphates	Phospho- glyceric acid	Phospho- pyruvic acid	Pyruvic acid	Lactic acid
1	(Normal blood) (No inhi- bitor)		-23 -100	+1 66 +0 79	+1 16 +6 99	+1 19 -3 81	+0 24 -0 05	+0 65 -0 01	+0 66 +4 47	nıl -1 29	nıl nıl	+1 51 +5 19	nıl +115
3	Iodoacetic acid (Controls)	1 08 × 10 ³	+4 -100	-0 42 +1 05	+7 90 +9 32	+10 22 -0 17	+0 70 +0 19	+0 23 +0 15	+4 67 +5 96	+1 00 -1 10	+1 07 nıl	+2 01 +6 04	+6 +143
2	NaCN (Controls)	4 08 × 10 ⁻³	-60 -100	-0.93 + 0.66	+4 26 -0 92	-4 13 -9 80	-0 07 -0 30	+0 61 -0 25	+0 53 +0 40	-156 -110	+5 84 nıl	$\begin{vmatrix} -0.42 \\ +5.02 \end{vmatrix}$	+90 +69
3	Quinine (Controls)	5 05 × 10 4	-42 -100	+2 39 +0 61	+5 97 +9 93	+1 14 -3 47	$-0.18 \\ -0.11$	+0 41 -0 02	+1 87 +5 69	-0.28 -1.60		+5 37 +4 44	+94 +118
3	Mepacrine (Controls)	3 94 × 10-4	-79 -100	+6 46 +0 61	-0 91 +9 93	+2 26 -3 47	-0.17 -0.11	+0 47 -0 02	+3 48 +5 69	-0 30 -1 60	nıl nıl	+1 02 +4 44	+57 +118

DISCUSSION

Although the malaria parasite utilizes glucose, its metabolism of this substrate is comparatively slow, thus, in whole blood suspensions, sufficient indigenous glucose is present to last the parasites for several hours, and added glucose produces no stimulation of respiratory activity. That glucose is a necessary substrate for the full activity of plasmodia is, however, shown by the fact that infected red cells lose 60 per cent of their respiratory activity when washed free from glucose, and that most of this lost activity can be restored by adding glucose to the washed cell suspension

The respiration rate of washed, parasitized red cells can be stimulated by substrates other than glucose. As observed by previous workers (Speck, Moulder and Evans, 1946), lactic and pyruvic acids are as active as, or slightly more active than, glucose in this respect (see, however, the next paragraph). Succinic and fumaric acids show a small but definite stimulatory effect. From these observations it is apparent that the carbohydrate metabolism of plasmodia proceeds to some end-point beyond lactic and pyruvic acids. The stimulation by succinate and fumarate suggests that pyruvic acid is metabolized via the Krebs citric acid cycle. Using parasites freed from red cells, Speck,

Moulder, and Evans (1946) obtained much greater stimulation with succinate

Analysis of parasitized red cell incubates for phosphorylated intermediates confirmed the view of previous workers that the initial stages of glucose metabolism by plasmodia follow the so-called Embden-Meyerhof-Parnas system characteristic of yeast and muscle metabolism. Since considerable quantities of lactic acid accumulated in these incubates, it seems that the conversion of glucose to pyruvic acid is more rapid than the subsequent utilization of pyruvate, the excess pyruvate being reduced to lactate. McKee et al. (1946) reported that lactate was produced six times as rapidly as it was utilized.

The amount of oxygen utilized by plasmodia bears no constant relation to the amount of glucose used. In parasitized red cell incubates respiring in the presence of glucose, the amount of oxygen consumed is at first less than one molecule per molecule of glucose used, but gradually increases to more than one molecule during the incubation period. This gradual increase in oxygen consumption probably indicates the "starting up" of additional oxidation systems as the metabolism of the initial quantity of glucose reaches the various stages. The fact that it takes several hours to build

up to the full rate of oxygen consumption emphasizes the sluggishness of carbohydrate metabolism in plasmodia

Oxygen enters into the metabolic system of plasmodia at two points at least, firstly in the oxidation of glucose to pyruvic acid, and secondly in the subsequent oxidation of the pyruvate Since the first oxidation (which takes place at the conversion of phosphoglyceraldehyde to phosphoglyceric acid) is inhibited by iodoacetic acid, it is concluded that it takes place through a chain of reducing reactions catalysed by dehydrogenase systems However, the final connection with atmospheric oxygen is probably made through the cytochrome oxidase system, since glucose oxidation is as powerfully inhibited by cyanide as pyruvate oxidation Furthermore, in the presence of cyanide, glucose and the phosphorylated intermediates are rapidly converted to lactic acid, which is an anaerobic reaction requiring no oxygen Pyruvate oxidation is not appreciably inhibited by iodoacetic acid, but is strongly inhibited by cyanide, and is therefore entirely dependent on the cytochrome oxidase system

Considerable quantities of glucose-1-phosphate (an intermediate of glycogen, but not of glucose, metabolism) accumulated in parasitized red cell incubates. This accumulation probably resulted from a "feed-back" from glucose-6-phosphate through the reversible phosphoglucomutase reaction. This view is strengthened by the observation that accumulation of glucose-1-phosphate was increased in the presence of iodoacetic acid, where the later stages of normal glucose phosphorylation were blocked.

Parasitized red cells from which all traces of indigenous glucose have been removed by repeated washing still show an appreciable oxygen uptake, which is still proceeding after 6 hours' incubation It is generally believed that plasmodia can metabolize substrates other than carbohydrate, but, in view of the slowness of glucose metabolism, the possibility cannot be ignored that sufficient quantities of the glucose intermediate compounds might remain in the parasites or red cells after washing to account for the "residual" respiration observed The oxygen uptake of washed, parasitized red cells was not stimulated by amino-acids (glutamate, aspartate or tyrosine) nor could any change in nonprotein or ammonia nitrogen be detected in the incubates This is not, however, in agreement with the results of previous workers (Moulder and Evans, 1946), who found both that amino-acids were utilized, and that non-protein and ammonia nitrogen were produced by plasmodia

Having obtained some indication of the course of carbohydrate metabolism in plasmodium, we may consider at what points in this system quinine and mepacrine exert inhibitory actions. In agreement with the observations of previous workers (Silverman et al., 1944) it was noted that neither drug, in low concentration, inhibited the oxygen uptake of parasitized red cells until it had been in contact with the cells for 2-6 hours, even then, the inhibition was not very pronounced

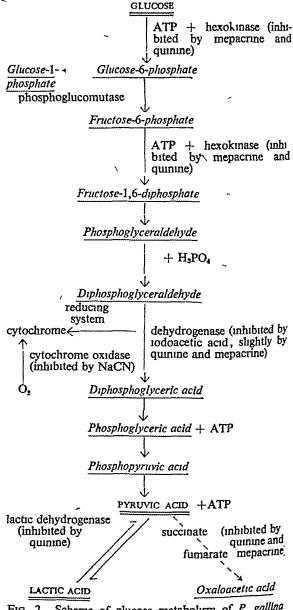


Fig 2—Scheme of glucose metabolism of *P gallina ceum*, with indications of the points of action of quinine and mepacrine

Bovarnick et al (1946b) reported that mepacrine inhibition of plasmodia could be reversed by ATP, and Speck and Evans (1945b) showed that both quinine and mepacrine inhibited yeast hexokinase In the present investigation, the analyses showed that, while quinine inhibited glucose utilization to a greater degree than menacrine, the latter compound showed a more pronounced inhibition of In the presence of mepacrine, the accumulation of glucose-1-phosphate was inhibited, presumably because of the formation of glucose-6phosphate was reduced Both drugs showed some iodoacetate-like inhibitory effects, but these were not well marked Quinine caused lactate and pyruvate to accumulate, and therefore inhibited lactate as well as pyruvate oxidation These possible points of action are indicated in Fig 2

From the above observations, it is clear that quinine and mepacrine exert inhibitory activity at several points in the metabolic scheme of plas-With an organism possessing a comparamodia tively complicated metabolism, it is necessary that a therapeutic agent should have this capacity for multiple attack in order to be effective against the organism For instance, trivalent arsenical compounds, which inhibit only one type of enzyme, the so-called -SH enzymes, are active against certain trypanosomes which possess a simpler type of metabolism, but are inactive against plasmodia, in which alternative metabolic paths probably exist, or a greater variety of substrates can be utilized The present investigation has given indications of the points of action of antimalarial drugs in one metabolic system — carbohydrate metabolism Further studies are necessary to find what proportion of the total inhibitory activity takes place at the different points, and, indeed, whether the greater part of the inhibition does take place against the carbohydrate metabolism, or against other metabolic functions

SUMMARY

1 Investigations on the oxygen uptake of *Plasmodium gallinaceum* show that washed, parasitized chick red cells can oxidize glucose, glycerol, lactate, and pyruvate, but not glutamate, aspartate,

or tyrosine Stimulation of oxygen uptake by succinate and fumarate indicates that pyruvate metabolism proceeds via the Krebs citric acid cycle

- 2 Observation of the changes in phosphorylated intermediates in washed, parasitized red cell incubates confirms the view that glucose metabolism in plasmodia proceeds *via* the Embden-Meyerhof-Parnas system characteristic of yeast and muscle metabolism
- 3 The possibility of metabolic systems in plasmodia other than carbohydrate is discussed
- 4 Quinine and mepacrine exert inhibitory activity at several points in the glucose metabolism of plasmodia. Quin ne inhibits hexokinase and phosphoglyceraldehyde dehydrogenase moderately, and possibly lactic dehydrogenase and pyruvate oxidation. Mepacrine inhibits hexokinase strongly, phosphoglyceraldehyde dehydrogenase moderately, and probably pyruvate oxidation.

The author is indebted to Mr L G Goodwin (Pharmacology Dept) for supplying chicks infected with *P gallinaceum*, and to Messrs P A Hankin and R W Neville for valuable technical assistance

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THE GLUCOSE METABOLISM OF TRYPANOSOMA EVANSI AND THE ACTION OF TRYPANOCIDES

BY

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(Received May 22 1947)

It has long been recognized that trypanosomes are dependent on an adequate supply of glucose for the maintenance of their metabolic processes (Yorke, Adams, and Murgatroyd, 1929, Geiger, Kligler, and Comaroff, 1930, von Brand, 1933) Later work has given some indication of the mechanism of the glucose metabolism of various species of the parasite, and the nature of the endpoints formed Reiner and Smythe (1934) showed that the end-point of glucose metabolism in T equiperdum was pyruvic acid, and the same group of workers (Reiner, Smythe, and Pedlow, 1936) showed that T lewisi metabolized glucose more completely to yield formic, acetic and succinic icids, ethyl alcohol and carbon dioxide and Stevens (1945) reported that the products of metabolism of T rhodesiense were succinic, pyruvic, lactic, acetic and formic acids, glycerol, ethyl alcohol, and carbon dioxide

Regarding the respiratory activity of trypanosomes, Christophers and Fulton (1938) showed that oxygen consumption by T rhodesiense was entirely dependent on the presence of glucose, and that 1 molecule of glucose required 1 molecule of They also demonstrated the presence of dehvdrogenase systems in trypanosome metabolism, and the absence of inhibition by cyanide Reiner and Smythe (1934) reported that T^{-} equiperdum produced very little carbon dioxide in the absence of bicarbonate, and that 180 molecules of acid (mostly pyruvic) were produced from the metabolism of 1 molecule of glucose These workers also showed that trypanosomes could metabolize glycerol as well as glucose, but only under aerobic conditions More recent work by Searle and Reiner (1940, 1941) has demonstrated the importance of carbon dioxide as an activator of anaerobic glycolysis in trypanosomes

These investigations show clearly the nature of the end-products of glucose metabolism in various species of trypanosomes, but give little indication of the intermediate processes by which these products are formed Indirect evidence, such as the ability of glycerol to replace glucose, suggests that the intermediate metabolism of trypanosomes follows the typical phosphorylation course associated with yeast and muscle metabolism. However, only in one instance, a recently published paper by Chen and Geiling (1946), has any direct evidence of glucose phosphorylation been demonstrated. These workers have shown that lysed trypanosomes will transform glucose to fructose-1,6-diphosphate and triose phosphates, and will oxidize phosphoglyceraldehyde to phosphoglyceric acid

The investigations described in this communication corroborate the findings of other workers regarding the respiratory activity of trypanosomes, and provide further evidence that glycolysis proceeds via the typical chain of phosphorylation reactions. Some preliminary indications are given of the points of attack by trypanocidal agents, on which there are no previous reports

MATERIALS AND METHODS

The organism was a strain of *Try panosoma evansi* isolated from a camel in the Sudan in 1938, and maintained in mice, in which it produces heavy, acute blood infections. Infected blood was obtained from the exposed hearts of mice killed by placing them in an atmosphere of carbon dioxide.

Respiration experiments -Oxygen consumption was measured in conventional Warburg constant volume respirometers, using 15 ml flasks maintained at 38° C A set of twelve respirometers was run in duplicate, triplicate, or quadruplicate groups, according to the number of variants required Since normal mouse blood showed only a negligible oxygen uptake, whole blood containing the trypanosomes was usually used, diluted with "isotonic buffer" (0 85 per cent NaCl, 100 ml, M/15 phosphate buffer, $p\hat{H}$ 73, 30 ml) containing a little citrate to prevent clotting concentration of trypanosomes was 10 to 100 million In a few experiments where blood-free per flask trypanosomes were required, the parasites were separated by adding an equal volume of distilled

water to the blood-buffer suspension, which contained 0.05 per cent glucose. The resultant lowering of tonicity lysed the red cells of the blood, but did not disrupt the trypanosomes, which were centrifuged down, washed free from haemoglobin with isotonic buffer-glucose, and finally resuspended in glucose-free isotonic buffer.

Glucose was determined in the incubates from the respirometer flasks by the method of Folin and Wu (1920) Pyruvic acid was determined by the modified method of Lu (1939) described by Umbreit, Burris, and Stauffer (1945), and lactic acid by the method of Barker and Summerson (1941)

Experiments on glucose phosphorylation—Mouse blood heavily infected with trypanosomes was diluted with isotonic buffer containing a known concentration of glucose. An aliquot of the suspension was deproteinized immediately by adding an equal volume of ice-cold 12 per cent (w/v) trichloroacetic acid. Similar aliquots were incubated at 38° C for 40-100 min, either alone, or in contact with inhibitors or trypanocides. These were then treated with trichloroacetic acid, chilled in the cold-room, and the precipitated protein removed by centrifuging. The concentration of trypanosomes was 10 to 100 million per aliquot.

The clear supernatants were subjected to a complete analysis for glucose, phosphorylated intermediates, pyruvic and lactic acids. Glucose was determined in dilutions of the supernatants by the method of Folin and Malmros (1929), pyruvic and lactic acids by the methods already referred to. The remaining supernatant was adjusted to pH 8 2 and analysed for hexose phosphates, triose phosphates, phosphoglyceric acid and phosphopyruvic acid using the experimental technique described by Umbreit, Burris, and Stauffer (1945). The amounts of the various intermediates were expressed in molecular proportions (μ moles)

The trypanocides investigated were phenylarsine oxide—trivalent arsenical, tryparsamide—pentavalent arsenical, undecane diamidine—straight chain diamidine, stilbamidine—aromatic diamidine

_Samples of phenylarsine oxide and tryparsamide were kindly supplied by Dr T Dewing of the Wellcome Chemical Laboratories, Dartford

RESULTS

Respiration experiments

Fig 1 shows the oxygen uptake of an infected blood suspension containing increasing concentrations of glucose. The initial respiratory rate was the same for all concentrations, but the oxygen uptake ceased abruptly as soon as all the glucose had been used up. This experiment showed clearly the dependence of trypanosomes on an adequate supply of glucose. The more gradual falling off of respiratory rate in the presence of excess glucose was at first presumed to be the result of toxic action by accumulated end-products. However, subsequent experiments showed that the pH of the

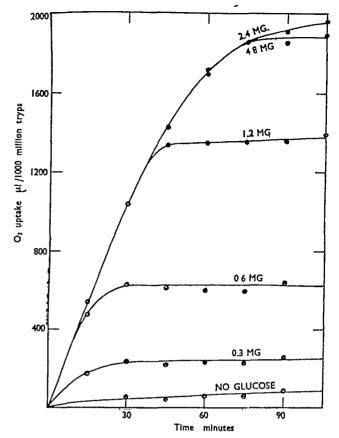


Fig 1 —Effect of increasing amounts of glucose on the duration of respiration of mouse blood suspension containing *T evansi*

medium was not appreciably lowered during the experiment, nor was added pyruvate (the chief end-product) toxic to the trypanosomes. The slowing down of respiratory rate may therefore be caused by exhaustion of available nutritional factors in the parasites or host blood.

The chief end-product of glycolysis by *T evansi* was pyruvic acid, which accumulated finally in the proportion of 1.75 molecules per molecule of glucose used, one molecule of oxygen being used up in the reaction (Fig. 2)

The rate of oxygen uptake of suspensions of trypanosomes separated from the blood of the host was reduced to 50 per cent of that of the original whole blood suspension. Nearly 75 per cent of the lost respiratory activity was recovered by adding 5 per cent of mouse plasma to the separated parasites (Table I), but addition of lysed mouse red cells had no stimulatory effect. It may be, therefore, that the trypanosome relies on the plasma of the host for many of the nutritional factors required for its metabolism. Individual factors, including pantothenic, nicotinic, adenylic acids, thiamin, riboflavin, pyridoxin, and inositol were

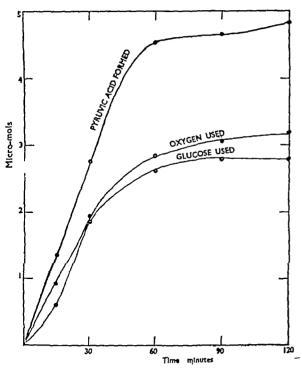


Fig 2—The utilization of glucose and oxygen and the formation of pyruvic acid by *T evansi* (whole blood suspension)

TABLE I

STIMULATORY EFFECT OF MOUSE PLASMA ON THE OXYGEN UPTAKE OF SEPARATED, WASHED TRYPANOSOMES

Washed parasites suspended in isotonic buffer + 0.05 per cent glucose O₂ uptake compared with that of the original whole blood suspension

Eτpt	% plasma added	% of "normal" respiration rate	% recovery
156	none 5 0	52 5 87 8	74 2
157	none 3 33 1 66 0 66 0 16	45 2 79 0 68 5 55 5 55 2	61 5 42 7 18 7 18 4

investigated, but none increased significantly the respiratory rate of separated trypanosomes

Trypanosome suspensions respired in the presence of glycerol as readily as with glucose One molecule of oxygen was used up per molecule of glycerol, and one molecule of pyruvic acid was formed Lactate, fumarate, succinate, glutamate, and aspartate were not metabolized Pyruvate produced a small additional oxygen uptake, par-

ticularly in the presence of glucose With glycogen, a slow, prolonged oxygen uptake was observed It was found, however, that glycogen was slowly hydrolysed to glucose by normal mouse blood. The respiration of the trypanosomes in the presence of glycogen therefore resulted from the utilization of this glucose as it was produced, and not from a direct metabolism of the polysaccharide.

TABLE II

EFFECT OF SPECIFIC INHIBITORS ON THE O2 UPTAKE

OF TRYPANOSOMES

Whole blood suspensions containing added

plucose

	Conce	%	
Inhibitor	μg /3 ml molar		inhibition
Iodoacetic acid	50 0	8 98 × 10 ⁻¹	92 0
	10 0	1 79 × 10 ⁻¹	89 3
	2 0	3 58 × 10 ⁻¹	53 3
	0 4	7 71 × 10 ⁻¹	4 8
	0 08	1 43 × 10 ⁻⁷	nil
Sodium fluoride	50 0	3 97 × 10 ⁻⁴	41 5
	10 0	7 94 × 10 ⁻⁴	13 0 -
	2.0	1 59 × 10 ⁻⁸	2 7
	0 4	3 18 × 10 ⁻⁴	nil
Sodium cyanide	50 0	3 4 × 10-4	10 3
	500 0	3 4 × 10-3	8 1

Effect of specific inhibitors—The inhibitory effects of iodoacetic acid, NaCN, and NaF on the oxygen uptake of infected blood suspensions are shown in Table II Iodoacetic acid and NaF were powerful inhibitors of trypanosome respiration Cyanide had little effect on the oxygen uptake, but the amount of pyruvic acid formed was reduced to one molecule per molecule of glucose (Fig 3)

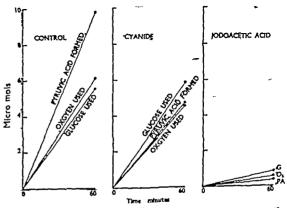


FIG 3—Effect of NaCN and iodoacetic acid on the utilization of glucose and oxygen and the formation of pyruvic acid by T evansi (whole blood suspension)

Effect of trypanocides—The oxygen uptake of trypanosome suspensions was almost completely inhibited by concentrations of 1 in 300,000 of phenylarsine oxide, but was only slightly inhibited by 1 in 30,000 of tryparsamide—Stilbamidine had little inhibitory effect even in concentrations as high as 1 in 6,000—Undecane diamidine, however, produced 50 per cent inhibition of oxygen uptake at 1 in 30,000 concentration, but no further inhibition was obtained on increasing the concentration above this level (Table III)

Phosphorylated intermediate analyses

In these experiments, the standard of activity was taken as the amount of glucose utilized by uninhibited trypanosome suspensions, and the changes in concentration of the intermediate and end-products were expressed as the mean number of molecules used (—) or produced (+) per 100 molecules of glucose consumed by uninhibited, infected blood suspensions

Glucose phosphorylation by normal and infected blood—Table IV shows that trypanosome-infected blood used more than 10 times as much glucose as did normal blood, that glucose-6-phosphate was

TABLE III

effect of trypanocides on the $o_{\underline{\mathbf{z}}}$ uptake of trypanosomes

Whole blood suspensions containing added glucose

T	Conce	%	
Trypanocide	μg /3 ml	molar	inhibition
Phenylarsine oxide (Trivalent As)	10 0 5 0 2 0 0 5 0 1	1 98 × 10 ⁻⁴ 9 93 × 10 ⁻⁴ 3 97 × 10 ⁻⁴ 9 93 × 10 ⁻⁷ 1.98 × 10 ⁻⁷	81 3 49 0 34 0 14 6 5 7
Tryparsamide (Pentavalent As)	100 0 10 0	1 09 × 10-4 1 09 × 10-4	1 70 4 78
Stilbamidine	500 0 100 0 100 0 10 0	6 32 × 10 ⁻⁴ 1 26 × 10 ⁻⁴ 1 26 × 10 ⁻⁴ 1 26 × 10 ⁻⁴	3 65 nıl 2 98 2 08
Undecane diamidine di-HCl	500 0 100 0 100 0 100 0	5 35 × 10 ⁻⁴ 1 07 × 10 ⁻⁴ 1 07 × 10 ⁻⁴ 1 07 × 10 ⁻⁴	48 5 51 5 48 0 32 4

TABLE IV

EFFECT OF SPECIFIC INHIBITORS AND TRYPANOCIDES ON GLUCOSE PHOSPHORYLATION BY T evansing Figures denote the mean numbers of μ moles of the substrates formed (+) or utilized (-) per hour per 100 μ moles of glucose consumed by the control suspension (uninhibited trypanosome-blood suspension)

	of glacost consumed by the control suspension (analysis of the suspension)												
No of expts	Inhibitor	Molar conc of inhibitor	Glucose	АТР	Glucose-1- phosphate	Glucose-6- phosphate	Fructose-6- phosphate	Fruct1, 6- diphosphate	Triose phosphates	Phospho- glyceric acid	Phospho- pyruvic acid	Pyruvic acid	Lactic acid
1	(Normal blood)		-7	+29	+12	-08	+049	+0 34	+03	-19	+0 18	+4	+13 7
4	(No inhib)		-100	+66	+14	-267	+0 79	-0 17	+107	-41	+3 65	+174	-0 21
4	Iodoacetic acid (Controls)	5 38 × 10 4	-38 -100	-12 +66	+22 +14	-13 8 -26 7	+0 42 +0 79	+0 38 -0 17	-20 +107	-11 -41	+2.55 +3 65	+12 +174	nıl 0 21
2	NaCN (Controls)	4 08 × 10 ³	-173 -100	+91 +58	-06 -06	-16 8 -20 3	+0 69 +1 23	-0 02 -0 01	+05 -01	+02 -29	+1 53 +3 65	+203 +159	+07 -38
2	Phenylar- sine oxide (Controls)	5 95 × 10 4	-24 -100	+108 +74	+41 +34	-20 9 -33 1	+0 07 +0 36	-0 15 -0 35	+28 +215	-20 8 -5 4		+7 +189	+35 +33
1	Stilbami- dine (Controls)	3 79 × 10 ⁻⁴	-78 -100	-41 +115	-107 +68	+105 -12	+0 96 +0 72	-0 70 -0 70	+64 +210	-89 -57		+202 +215	+88 +123
2	Undecane diamidine (Controls)	64 × 10-4	-148 -100	+72 +58	-07 -06	-94 -203	+1 36 +1 23	+0 38 +0 01	+32 -01	-07 -29	+5 36 +3 65	+190 +159	-06 -38

very rapidly utilized by infected blood, and that glucose-1-phosphate, triose phosphate, phosphopyruvic acid and particularly pyruvic acid accumulated in parasitized blood incubates. Adenosine triphosphate (ATP) accumulated more rapidly in infected than in normal blood. The mean amount of pyruvic acid formed was 1.74 molecules per molecule of glucose used, which agrees with the results of the respiration experiments. Normal blood produced practically no pyruvic acid, but some lactic acid accumulated.

Effect of specific inhibitors—In the presence of iodoacetic acid, glucose utilization was strongly inhibited, ATP was consumed, and glucose-6-phosphate disappearance was retarded, while hexose diphosphate accumulated. These observations are in accordance with the accepted view that iodoacetic acid blocks the dehydrogenase reaction which promotes the oxidation of phosphoglyceraldehyde to phosphoglyceric acid. Cyanide actually increased the amount of glucose utilized, but reduced the proportion of pyruvic acid formed. It had little effect on the intermediates of the system.

Effect of trypanocides—Of the trypanocides investigated, phenylarsine oxide had the most striking effect. In the presence of this compound, very little glucose was utilized, and ATP accumulated in the incubate. This suggests an inhibition of hevokinase, since the ATP released by dephosphorylation was not being re-utilized to phosphorylate more glucose. The intermediates beyond the "block" were rapidly consumed

Undecane diamidine behaved similarly to cyanide in that it increased the glucose utilization and reduced the proportion of pyruvic acid formed Stilbamidine inhibited glucose utilization slightly, but increased the proportion of pyruvic acid produced to 26 molecules. Its effect on the intermediate metabolism resembled that of iodoacetic acid, though glucose utilization was not inhibited to the same extent.

DISCUSSION

The preliminary observations on *T evansi* showed that suspensions of the parasites metabolized glucose and glycerol exclusively, the oxygen uptake ceasing abruptly as soon as the added substrate was consumed. The end-point of the metabolism of either substrate was pyruvic acid, and quantitatively the gross reactions taking place conformed fairly closely to the equations

and $C_3H_4O_3 + O_2 \longrightarrow C_3H_4O_3 + 2H_2O_3$ glycerol

These observations are similar to those reported for T equiperdum (Reiner and Smythe, 1934) No other substrates were utilized by T evansi Polysaccharides (glycogen) were not broken down directly, and none of the intermediates associated with the subsequent utilization of pyruvic acid (succinate and fumarate), nor amino-acids, stimulated the respiratory activity of the trypanosomes. The metabolic activity of this trypanosome therefore appears to be limited to the conversion of hexoses to pyruvic acid, since glycerol can be regarded as an intermediate of this process

The next step was to examine the intermediate stages of this reaction In most living cells, glucose breakdown proceeds via the so-called Embden-Meyerhof-Parnas scheme of reactions (Fig. 4) which operates through a chain of phosphorylated hexose Previous work on trypanosomes intermediates suggested that the metabolism of these organisms followed this scheme In the present work, analysis of trypanosome suspensions before and after a period of incubation with glucose showed that active changes in the levels of phosphorylated intermediates had taken place These changes were much greater in parasitized blood than in normal mouse blood Addition of iodoacetic acid slowed down the rate of utilization of the hexose phosphates as well as the oxygen consumption of the parasites From these observations it is concluded that the glucose metabolism of the trypanosomes followed the Embden-Meyerhof-Parnas scheme

The only point at which atmospheric oxygen enters into the above scheme is in the oxidation of phosphoglyceraldehyde to phosphoglyceric acid Biological oxidations may be accomplished either by transferring oxygen through a chain of oxidation reactions to the compound to be oxidized, or by transferring hydrogen from the compound through. a chain of reducing actions to a point where it can be oxidized to water by atmospheric oxygen dation systems (usually including the cytochrome system) are inhibited by cyanide, dehydrogenase systems by iodoacetic acid Since the oxygen up take of trypanosomes was inhibited by iodoacetic acid, but not by cyanide, it is concluded, in agreement with Christophers and Fulton (1938), that only dehydrogenase systems operate in their utilization of oxygen

In any metabolic system, certain co-factors are necessary for the functioning of the enzyme reactions involved. The factors required by trypanosomes may be obtained from the plasma of the

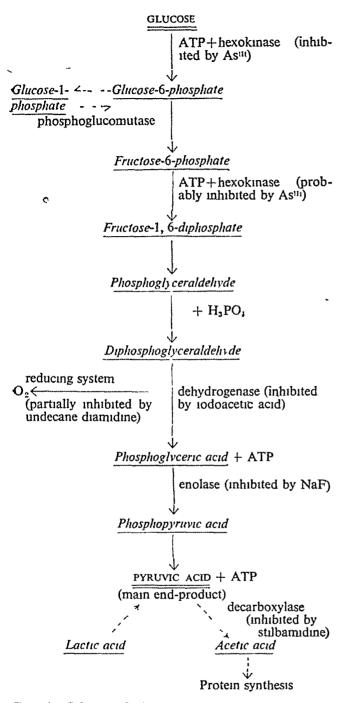


Fig 4—Scheme of glucose metabolism of *T evansi*, with indications of the points of action of trypanocides

host, since the respiration rate of trypanosomes separated from plasma was reduced by half, and the activity could be restored by adding normal mouse plasma. Other explanations, such as osmotic disturbances and surface phenomena, could account for this loss of metabolic activity, but the fact that only 5 per cent of plasma was required to restore most of the lost respiratory rate would

favour the nutritional factors as the most likely explanation

Although the above equation for the oxidation of glucose to pyruvic acid was fairly well satisfied, it was noticed repeatedly that only 1.75 molecules of pyruvic acid accumulated, instead of the theoretical 2 molecules, and that a little more than one molecule of oxygen was utilized. This suggests that a little of the pyruvate is oxidized by the trypanosomes, and might be the source of the protein which must be synthesized for the multiplication of the parasites Some indication of the mode of metabolism of the quarter molecule of pyruvic acid was provided by the observation that cyanide decreased the amount of pyruvic acid which accumulated, relative to the amount of glucose Tauber (1938) reported that cyanide was an activator of co-carboxylase, and it is probable, therefore, that the pyruvate undergoes preliminary decarboxylation The fact that trypanosomes produce a small amount of respiratory CO₂ (Reiner and Smythe, 1934, Christophers and Fulton, 1938) supports this theory, since the reaction ending in pyruvic acid produces no CO₂

It is now possible to consider at what points in this metabolic scheme the various types of trypanocides exert their action. It is clear from the results that trivalent arsenicals strongly inhibit the hexokinase reaction, and therefore block the utilization of glucose at the first stage, conversion to the 6-phosphate. This observation agrees with the report of Dixon and Needham (1946) on the action of arsenical vesicants on the glucose metabolism of skin. Pentavalent arsenicals showed no inhibition of trypanosome respiration, which is in accordance with the view that these compounds must first be reduced in vivo to the trivalent state before becoming active.

The point of action of the diamidines was less obvious than that of arsenic, but it appears that the straight chain compounds (undecane diamidine) differ in action from the aromatic compounds (stilbamidine) The former compound behaved similarly to cyanide in decreasing the amount of pyruvate formed, while stilbamidine had the reverse effect of increasing the pyruvate stilbamidine Though accumulation striking changes in the levels of phosphorylated intermediates, it did not interfere to any extent with the utilization of glucose or oxygen during the 60 to 90 minute period of the manometric experiments Its trypanocidal action may therefore result from the inhibition of pyruvate metabolism, causing ultimate suppression of growth These conclusions are in agreement with the observations of Lourie and Yorke (1937) that this type of compound (synthalin) only showed trypanocidal activity in vitro after at least 24 hours' incubation The point of action of undecane diamidine is not clear, since intermediate glucose metabolism is not greatly influenced by the compound It did, however, cause a 50 per cent reduction in oxygen uptake, so that its point of action may lie somewhere in the dehydrogenase system (Fig. 4)

Two compounds which do not appear to fit directly into the metabolic scheme of trypanosomes require some consideration. The first one, glucose-1-phosphate, is not an intermediate of glucose but of glycogen phosphorylation Although it was shown that trypanosomes do not metabolize glycogen directly, an increase in glucose-1-phosphate was observed in some cases in uninhibited incubates and in the presence of inhibitors blocking the normal metabolic chain In 10doacetic acid inhibition, where the blockage occurs beyond the glucose-6-phosphate stage (Fig 4), accumulation of glucose-1-phosphate might result from a "feedback" from the 6-phosphate via the reversible phosphoglucomutase reaction With phenylarsine oxide, however, where the greatest accumulation of glucose-1-phosphate occurred, this "feed-back" could not operate, since the formation of the 6phosphate itself from glucose was inhibited accumulation of the 1-phosphate in this case cannot as yet be explained

The second compound for consideration is lactic acid, which accumulated in normal blood incubates, but not in incubates containing trypano-Moreover, when glucose metabolism was blocked by iodoacetic acid, lactic acid initially present disappeared during incubation observations suggest that indigenous lactic acid can be converted to pyruvic acid by the parasites Further evidence of this possibility is provided by the observed accumulation of more than the theoretical two molecules of pyruvic acid in the presence of stilbamidine

SUMMARY

1 Investigations of the oxygen utilization of suspensions of T evansi show that this trypanosome metabolizes glucose mainly to pyruvic acid, though a small quantity of this end-product is probably further utilized by decarboxylation

- 2 Analysis of trypanosome suspensions for phosphorylated intermediates in the presence of specific inhibitors shows that the intermediate metabolism of the parasites follows the typical Embden-Meyerhof-Parnas scheme characteristic of yeast and muscle metabolism
- 3 At least some of the nutritional factors required in this metabolism may be obtained from the plasma of the host
- 4 Trypanosomes appear to be able to utilize the lactic acid in the host blood, probably by conversion to pyruvic acid
- 5 Observation of the effects of typical trypanocides suggests that trivalent arsenicals inhibit the hexokinase reaction in trypanosomes, that pentavalent arsenicals are inactive unless reduced in vivo to the trivalent state, that straight chain diamidines (undecane diamidine) partially inhibit the dehydrogenase system on which oxygen transport depends in trypanosomes, and that aromatic diamidines (stilbamidine) probably inhibit the decarboxylation of pyruvic acid

The author is indebted to Messrs P A Hankin and R W Neville for valuable technical assistance

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SULPHETRONE*: A CHEMOTHERAPEUTIC AGENT FOR TUBERCULOSIS PHARMACOLOGY AND CHEMOTHERAPY

BY

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The discovery and evaluation of the chemotherapeutic activity of diaminodiphenvisulphone in these laboratories (Buttle, Stephenson, Smith, Dewing, and Foster, 1937) inaugurated a search which has gone on ever since for a drug which combined the activity of the sulphone with freedom from toxicity to the host. A number of derivatives proved to be active antibacterial agents and were subjected to a comprehensive pharmacological study. The sulphone derivatives include in their range high antibacterial activities to streptococci, pneumococci, and *M tuberculosis*.

One derivative, 4 4'-bis(\gamma-phenyl-n-propylamino) diphenylsulphone-tetrasodium sulphonate, given the registered name "sulphetrone," attracted attention by reason of its freedom from toxicity and its high antibacterial activity. A pharmacological and therapeutic study of this derivative has been made and details are given of the structure, chemical and physical properties, pharmacology, experimental therapy, and possible clinical uses of the drug

CHEMISTRY

Preparation and properties of sulphetrone

The chemical preparation has been described by Gray and Henry (1936), and by Buttle, Dewing, Foster, Gray, Smith, and Stephenson (1938) The final stage is precipitation with alcohol which yields an amorphous material containing, when air-dried, 5-7 per cent water which it loses when heated to 110° C in vacuo Obtained crystalline, the compound contains 5-7 per cent of water, the calculated percentage for monohydrate is 198 Its probable constitution is

C.H.CH CH.CH NH C.H.SO.C.H.NH CH CH.CH C.H.

So.Na So.Na So.Na So.Na So.Na Molecular weight = 892 5

Typical migrating boundaries may be obtained by electrophoresis, and analysis by this means has enabled Mr P A Charlwood, of these laboratories, to say that the drug as prepared is not homogeneous but consists of a major component of some 94 per cent and a minor component of 6 per cent

The compound is insoluble in alcohol and other organic solvents, but is exceedingly soluble in cold water to give a syrup. Twenty and forty per cent (w/v) solutions are stable when neutral or slightly alkaline and may be autoclaved. Boiling with normal acid yields a brown coloured complex of high molecular weight and only by more drastic hydrolysis is it possible to recover a small proportion of the whole as diaminodiphenylsulphone.

Estimation of sulphetrone

Sulphetrone was estimated in blood, urine, cerebrospinal fluid and tissues by diazotization and coupling to N-(1-naphthyl)-ethylenediamine hydrochloride (Bratton and Marshall, 1939) and estimating the pigment colorimetrically or, preferably, absorptiometric-Although the facility with which derivatives of diaminodiphenylsulphone are adsorbed by undenatured protein is not shared by sulphetrone, admixture with precipitated proteins is intimate, and therefore the conditions governing the optimum recovery of drug were determined experimentally When the following conditions are rigidly adhered to, 90 per cent recoveries of drug are obtained, the over-all dilution of 1 in 15 and the concentration of acid are critical 05 cc blood, or other body fluid, is added to 5 cc N/1 HCl and mixed well, 20 cc 12 per cent (w/v) trichloroacetic acid are added, and the well-mixed solution filtered immediately through

^{*}The development of sulphetrone is part of the programme of work on antituberculous compounds carried out by the Therapeutic Research Corporation of Great Britain, Limited

a No 5 Whatman paper and repassed until brilliant, 3 cc of filtrate are mixed with 0.05 cc 0.3 per cent fresh (weekly) sodium intrite and left 3 min, 0.05 cc 1.5 per cent ammonium sulphamate is now added and the solution left 2 min, finally, 0.05 cc 0.1 per cent N-(1-naphthyl) ethylenediamine hydrochloride is added and mixed well. The colour should be allowed to develop for 30 min before being read colorimetrically or absorptiometrically with a Wratten 61 filter.

The visible absorption spectra of the naphthylethylenediamine derivatives of diaminodiphenylsulphone and sulphetrone are shown in Fig 1. The colour intensity of the sulphetrone-dye complex is much less than that of the parent substance, although the optimum conditions for colour production were found experimentally and the concentrations were adjusted to be approximately equivalent, this suggests that sulphetrone may not be readily hydrolysed, a suggestion borne out by the pharmacological evidence. The ultra-violet absorption spectra of the derivative and the parent compound are included in Fig 1.

All estimations of sulphetrone are given in terms of

the anhydrous compound

By collaboration with The Tintometer, Ltd, a standard Lovibond colour disc is available for the rapid reading of blood and body-fluid estimates falling within the range of 0 to 9 mg per 100 cc

TOXICITY

Acute toxicity

A 10 per cent (w/v) solution of sulphetrone is isotonic with 0.91 per cent sodium chloride, and hypertonic solutions up to 60 per cent (w/v) are readily obtained. The need to administer large volumes of these grossly hypertonic solutions, together with poor absorption from the gut and

low intrinsic toxicity, make the determination of acute toxicity in precise figures of questionable significance

The results of an estimate of oral toxicity are shown in Table I All mice receiving 20 g per

TABLE I
Toxicity for mice of sulphetrone given orally in solution

Dose g per kg	Dead/Total	Time of death in hours	Mean blood concentration at death, mg per 100 c c ± S D
2 00 1 75 1 50	20/20 12/20 4/20	0 75 1 50 within 5 00	960 ± 330 2700 ± 874 240 ± 10 9
1 25	0/20		Mean blood level of all survivors at 5 hours 14 ± 2

kg as 60 per cent solution, and those which died after 175 and 150 g per kg, died quickly, after collapse and circulatory failure Post mortem there was gross dehydration and collapse of major blood vessels, the stomach and gut were distended and the contents contained blood. The opinion was formed that the hypertonicity of the solutions made a major contribution to the death of the animals. For example, the blood concentrations at death after the three higher doses appear to be related to hypertonicity and time of death, and are in marked contrast to the concentration found at the fifth hour in the fourth group

Intravenous administration of a 20 per cent (w/v) solution caused deaths in groups of mice at

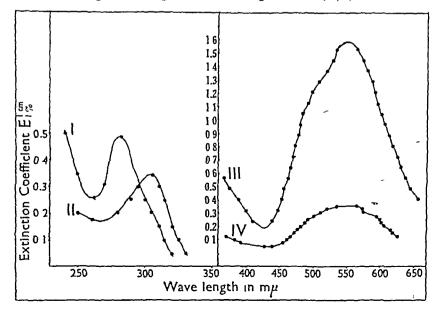


Fig 1-Spectrometic measurements of diaminodiphenylsulphone I and sulphetrone II in the ultra-violet, and of diaminodiphenyl - sulphonenaphthylethylenediamine dyeIII and sulphetrone naph thylethylenediamine dye IV in the visible wavelength extinction Ordinates, efficient, Elcm Abscissae. wavelength, in mµ I, 1018 mg per 100 c.c N/10 HCl, II, 1 022 mg in 100 cc H₂O, III, 025 mg per 100 cc N/1 HCl, IV, 08 mg per 100 cc N/1 HCl

doses of 25,275, and 3 g/kg but not at 15 g/kg, at which dose level the mean blood concentration was 650±160 mg per 100 cc Most deaths took place within one minute. The mice became cyanosed, involuntary spasms were overtaken by epileptiform convulsions which passed to extensor hind-limb paralysis, dyspnoea was followed by apnoea. There were a number of late deaths (1-2½ hours). The results are set out in Table II Computed by the method of Bliss (1938) the LD50 is 27 g with a range of 24 to 3 g per kg

TABLE II

Toxicity for mice of sulphetrone given intravenously in solution

Dose g 'kg	Dead/ Total	Time of death	Mean blood concentration at death, mg per 100 cc ± S D
2 5	6/20	4 in 1 min 2 in 2 5 hrs	1000 ± 200
2 75	14/20	10 in 1 min 4 in 1 hour	1150 ± 240
3 00	14/20	14 in 1 min	1400 ± 400

A dog was given 1 g sulphetrone per kg intravenously in 40 per cent (w/v) solution (a total of 10 g), the resulting blood concentrations were 61 8 mg per 100 cc at 15 min, 14 mg at 1 hour, 18 mg at 2 hours, and 6 8 mg at 24 hours. It will be seen that urmary clearance was rapid. Apart from lethargy and unsteadiness of gait, no symptoms were observed until after one hour, when the animal had a rigor with tachycardia lasting for 15 min. Two hours after the injection the animal was in high spirits

Intraperitoneal injection of 2 g per kg in 40 per cent (w/v) solution was followed by essentially the same symptoms and resulted in a blood level of 964 mg per 100 c c at 15 min, 18 mg at 1 hour, and 2 mg at 24 hours

After 2 g per kg intravenously the dog collapsed for a brief period 20 min after the injection, 20 min later there was a rigor and vomiting and a period of tachycardia for 15 min. The animal was lethargic but recovered, and appeared normal in 2 hours. Blood levels were 160 mg per 100 c c at 15 min, 38 mg at one hour, and 25 mg at 24 hours.

Experiments on the effect on alkali reserve, described later, suggest that some but not all of the immediate effects after large intravenous doses may be associated with increased plasma alkali However, since blood concentrations of the simple sulphonamides of half this magnitude cause severe symptoms and death in dogs, it is apparent that sulphetrone has little acute toxicity in the sense in which this term is normally used

Chronic toxicity

Four adult rabbits of mixed sexes weighing 25-3 kg were kept in metabolism cages for 40 days, during which period they received a diet containing 4 per cent sulphetrone and consisting of bran, sugar-beet pulp, and water Two additional rabbits were fed upon the diet without drug

A record was made of the drug intake, together with daily estimations of the concentrations of drug in blood, urine, and faeces Estimations were made of reticulocytes, erythrocytes, total

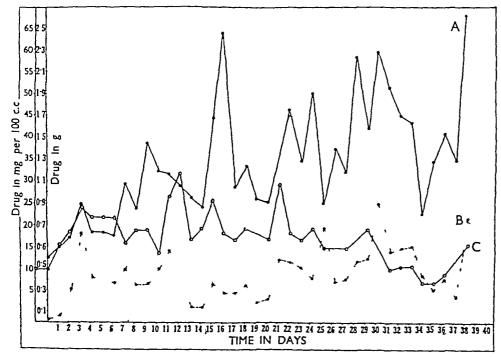


Fig 2 —Sulphetrone drugbalance experiments in a group of four rabbits receiving 4 per cent of drug in the diet A, average daily drug in urine and B, average daily drug in faeces, the ordinates being drug in grams C, average daily blood concentration, the ordinates being drug in per 100 c c Equilibrium is established after 3 or 4 Between days days 6 and 20, about onethird of the drug is unabsorbed, subsequently this figure drops to about onequarter

white-cell count, and haemoglobin concentration Although no abnormalities were detected in the white-cell count, progressive hypochromic anaemias were observed in 3 out of 4 rabbits. In one rabbit this was the cause of death at the 15th day. In Fig. 2 are graphed the mean amounts of drug in urine (A) and faeces (B) and the blood level (C) in mg per 100 c.c. Equilibrium was achieved after about three days. At first about 1/3, but later only about 1/4, was unabsorbed, After equilibrium had been achieved the blood level averaged 18 6 mg per 100 c c ±SD 60

Treatment of hypochromic anaemia in rabbits

During the first experiment on rabbits no abnormal excretion of blood pigments was observed Occasional (8) estimates of methaemoglobin by Zeiss pocket spectroscope were negative. Although the urine was darker in colour than usual no abnormal urobilin excretion was observed. Soon after the administration of the drug there was a high colour index, but this shortly fell, and the anaemia became hypochromic in type. Reduced haematopoiesis rather than abnormal degradation was suspected.

In a second experiment four adult rabbits, two male and two female, of 2-25 kg weight, were fed for 25 days with a diet of bran, sugar-beet pulp, water, and 4 per cent of sulphetrone addition to estimates of drug balance, daily estimates were made of reticulocytes, erythrocytes. haemoglobin, and urobilin Estimates of total urinary porphyrins excreted and of methaemoglobin were also made The observations are summarized in Fig 3 A slight though significant increase in urobilin occurs at once and is continued throughout the experiment, reticulocytosis indicates an attempt to make good the deficiency of cells, but an increase in colour index shows this This state corresponds to a slight but persistent haemolytic anaemia At this stage a precipitous anaemia with a raised colour index intervenes, this responds to iron, peroral or parenteral, but only to a limited degree

Absorption of the iron salt of sulphetrone

The fact that iron by mouth stimulated haematopoiesis suggested a direct interference by sulphetrone with iron metabolism. The iron salt was prepared by Mr W H Gray of the Wellcome Laboratories for Research in Tropical Medicine, and its absorption after oral doses was compared with sulphetrone. Given orally the iron complex is completely unabsorbed, parenterally it is distributed like sulphetrone. This finding taken with

the evidence of haematopoiesis after oral iron (Fig 3) indicates that sulphetrone combines with alimentary iron and prevents its absorption

Modification of intestinal flora by sulphetrone

At this stage, as a result of comparative tests with mice (Brownlee and Tonkin, 1941) the marked ability of sulphetrone to modify the intestinal flora was appreciated The extent to which many animals, and particularly ruminants, rely on biosynthesis for growth factors, some of which are vitamins, suggested a third experiment with the same diet but containing 10 per cent of dried yeast Four adult rabbits, male and female, weighing 25-3.5 kg were kept in metabolism cages for 39 days on the modified diet containing 10 per cent dried yeast. The drug balance figures were essentially similar to those of the first experiment. The animals maintained good condition and put on weight, at autopsy on the 45th day, no gross changes were seen in any of them Histology of spleen, kidney, and red bone-marrow showed a normal picture Slight cloudy swelling of some peripheral liver cells was the only abnormality observed The results of blood examination (Fig. 4) justify the conclusion that the gross anaemia previously observed arose directly from a lack of an essential metabolite previously furnished largely by biosynthesis which was prevented by unabsorbed sulphetrone residues and was now furnished by dried yeast. When this form of anaemia is excluded hypochromic anaemia attributable to iron lack is still present. On the third day after withdrawal of drug the residual haemolytic anaemia was made good

Specific toxic effects

It is known that sulphonamide drugs share with certain thioureas the property of causing hyperaemia and hyperplasia of the thyroid gland in some animals and in man, when they are administered at dose levels and for times similar to those in therapeutic use. The hyperplasia is believed

TABLE III —
Goitrogenic effect of drugs fed to groups of five littermate rats as 1 per cent of the diet, for 17 days

Groups			Devia- tion weight per cent	Thyroid hyper aemia	Thyroid weights mg per 100 g	
Control	47 2	97 5	103	+++	10 1	
Sulphetrone	45 7	92 0	100 5		13 6	
Sulphadiazine	48 0	88 0	83		17 6	
Sulphaguanidine	55 0	93 8	70		20 1	

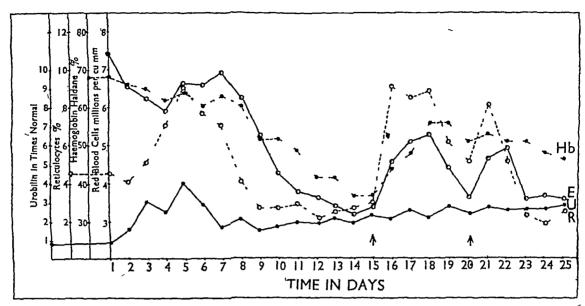


Fig 3 — Typical response of a rabbit (3353) to a 4 per cent sulphetrone diet. Hb is the haemoglobin estimate (Haldane), E, red blood cells in millions per cu mm, R, reticulocytes in per cent of red blood cells, U, spectrometric estimates of urobilin (Watson, 1936) in times normal At each arrow, 10 mg of iron was given by mouth, in the form of iron ammonium chelidamate

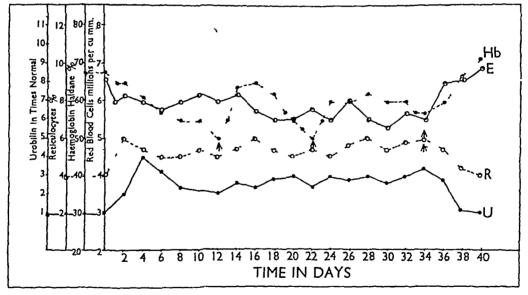


Fig 4—Typical response of a rabbit (3363) to a 4 per cent sulphetrone diet, but containing in addition 10 per cent of dried yeast. Hb, is the haemoglobin estimate (Haldane), E, red blood cells in millions per cu mm, R, reticulocytes in per cent of red blood cells, U, urobilin by spectrometric estimates of urobilin (Watson, 1936) in times normal. At each single arrow, 10 mg of iron, in the form of iron ammonium chelidamate was given by mouth. At the double arrow drug was withdrawn

(Astwood, Sullivan, Bissell, and Tyslowitz, 1943) to result from a hypothyroidism produced by failure to synthesize thyroid hormone. The goitrogenic activity decreases from sulphadiazine, sulphapyridine, sulphathiazole, sulphaguanidine, sulphanilylurea, sulphanilamide to sulphasuxidine (MacKenzie and MacKenzie, 1943)

Sulphetrone was tested for this specific toxic effect together with sulphadiazine and sulphaguanidine in litter-mate groups of five immature female rats of Wistar strain fed on a powdered diet containing 1 per cent of the drugs for a period of 17 days. The thyroids were removed, weighed, and studied histologically. The results summarized

in Table III show a decrease in goitrogenic activity from sulphaguanidine and sulphadiazine to sulphetrone, which shows a slight toxic effect

Effect on normal growth of rats

The effect of feeding a synthetic diet containing 1 per cent of sulphetrone and 0 01 per cent of iron to litter-mate groups of rats for 78 days is summarized in Table IV Both the female group and

TABLE IV

The effect on the growth-rate, and thyroid weights, of a 1 per cent sulphetrone diet fed to two litter-mate groups male and female, of rats, for 78 days Male and female control groups are included

Rats	Average	weight	Thyroid weights			
Litter mate groups of	in		mg per 100 g mean ± S D			
eight	Initial	After 78 days	пеан ± 5 Д			
Drug 1% o Controls o Drug 1% o Controls o	34.0 33 9 30 9 32 8	180 6 192 1 138 0 ,149 1	$\begin{array}{c} 9.2 \pm 0.8 \\ 8.9 \pm 0.7 \\ 11.5 \pm 1.1 \\ 9.9 \pm 0.8 \end{array}$			
		1 1				

the male group were retarded in growth for the first two weeks, but thereafter increased in weight by normal increments so that graphs of the growth-rates followed parallel courses. The weight increases of the thyroid glands are not significant in comparison with those produced by treatment with sulphonamides.

Effect on alkalı reserve

The evidence of acute and chronic toxicity together with the spectrometric evidence of the difference between the dye-coupled products of sulphetrone and of diaminodiphenylsulphone shows that sulphetrone is not hydrolysed to diaminodiphenylsulphone in the body. Since large amounts of the drug pass through the blood stream the probable effects of hydrolysis of one or more of the sodium sulphonate radicles on the alkali reserve were investigated. The results, which were unexpected are discussed later.

Groups of adult rabbits of mixed sex were maintained on a diet of bran and oats containing 50 per cent of water for one week to stabilize their metabolism, and were then given a single oral or parenteral dose of 1 g sulphetrone per kg as a 50 per cent (w/v) solution. At intervals blood was withdrawn from the heart into an oxalated syringe, immediately centrifuged under paraffin, and the CO. capacity determined by the volumetric method of Van Slyke. The results (Table V) show a rise in

TABLE V

Plasma CO₂—combining capacity, in volumes per cent, after oral and intraperitoneal doses of 1 g per kg sulphetrone in rabbits

Rabbit	CO ₁ capacity of plasma (vols %) after								
	0 hr	l hr	2 hr	4 hr	6 hr	24 hr	mg per 100 c c ± S D		
1 intraperitoneal 2 intraperitoneal 3 intraperitoneal 4 oral 5 oral	32 9 32 0 40 6 40 6 38 1	39 6 40 6 39 0 39 0 41 0	45 4 42 5 36 0 36 0 47 0	52 1 42 5 -40 3 40 3 48 5	44 3 47 4 40 6 40 6 40 3		75 ± 25 40 ± 32 		

the alkali reserve after an oral dose of sulphetrone in one of two experiments, and after an intraperitoneal injection in two of three experiments

The effect of parenterally administered drug is the same in the dog (Table VI) and it is interesting

TABLE VI

Plasma CO₂—combining capacity, in volumes per cent, after parenteral sulphetrone or sodium bicarbonate in dogs

Dog	co,	Mean blood concen- tration mg per					
-	0 hr	1 hr	2 hr	4 hr	6 hr	24 hr	100 c.c ± S D
A Sulphe trone 2g /kg I P	53 1	_	56 7	57 1	57 1	57 3	17 7±12
B Sulphe trone lg/kg I V	54 6	62 0	68 5	52 5	61 5	65 0	85±4
C Sulphe trone lg /kg I V D Sodium bi	43 5	46 5	490	81 0	52 0	_	22 0±16
carbonate 94 mg /kg I V E Sodium bi carbonate	40 9	48 9	50 4	48 1	<u> </u>	-	
188 mg /kg I V	43 6	55 1	52 0	53 5	51 7	_	

that the effect of 1 g sulphetrone per kg is approximately matched by 94 mg sodium bicarbonate per kg injected intravenously. This equivalence corresponds to the sodium of one of the four sodium sulphonate radicals being set free as sodium hydroxide. Dogs injected intravenously with this dose of sodium bicarbonate are subdued and

lethargic for about 30 minutes but show no other signs of toxicity.

The alkalı reserve after repeated doses of sulphetrone

For the purpose of a "chronic" test a total daily dose of 0.5 g sulphetrone per kg was given orally in 2 portions as a 5 per cent (w/v) solution Five adult rabbits fed on a diet of bran, sugar-beet pulp, and water were divided into groups, one of which received drug and the other water for a period of 7 days Eighteen hours after the last treatment the CO₂ capacity of the plasma was estimated. Treatment was now resumed so that rabbits which had been given water now received sulphetrone and vice versa The plasma CO, capacity, together with the blood concentrations of sulphetrone at the end of four weekly periods, are given in Table VII, which shows that when repeated doses are given from day to day equilibrium is achieved

TABLE VII

the CO, capacity of the plasma of the

The CO₂ capacity of the plasma of rabbits, as volumes per cent, after repeated doses of sulphetrone in a cross-over test

Rabbits		CO ₂ capacity of plasma as volumes per cent after							
	Water 5 c.c /kg -twice daily	Sulphetrone 5 c c. of 5% twice daily	of drug, mg per 100 c c						
5 6 7 8 9 10 11 12 13	55 0 28 5 46 0 47 0 41 5 46 0 37 0 50 0 53 0 49 5	33 0 44.5 48 0 35 0 59 0 51 0 39 5 — 51 5 41 0	10 7 6 0 8 0 8 8 9 0 5.2 8 8 - 3 0						
Mean value = S D	453 ± 81	11.7 ± 8 4	76 <u>=</u> 25						

ABSORPTION AND EXCRETION

When sulphetrone in solution was given by mouth to groups of mice the blood concentration—time curves were about the same for large and small doses. Typical results are given in Fig. 5, curves for intravenous and intraperitoneal injections being also included, curves for oral doses of diaminodiphenylsulphone and sulphanilamide are included for comparison.

A closer relation between large and small doses is seen in dogs after single doses given orally

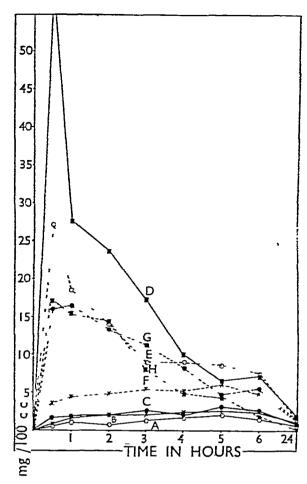


Fig 5—Blood concentration-time curves after sulphetrone, diaminodiphenylsulphone and sulphanilamide Each curve is the mean of determinations on five fasting mice A, sulphetrone, 0 1 g./kg by mouth B, same, 0 5 g./kg C, same, 1 0 g/kg D, same, 0 1 g./kg as 40 per cent solution intravenously E, same, 0 1 g./kg as 40 per cent solution intraperitoneally F, diaminodiphenylsulphone, 0 1 g./kg. suspended in acacia by mouth. G, same, 0 25 g./kg H, sulphanilamide, 0 1 g./kg suspended in acacia by mouth.

Blood and urine concentrations after intravenous doses are also included in Table VIII.

The total quantity excreted in the urine is given in per cent of the amount administered. It is interesting that although drug given intravenously is cleared from the blood stream rapidly and excreted almost completely in the urine in 24 hours, only 75 per cent of an oral dose is excreted in the same time. Evidence is given later that poor absorption from the bowel, and in particular from the large intestine, may account for the remainder

Blood concentration-time curves obtained in a dog after an oral dose and after the same dose given in three divided portions at three-hourly in-

Dose Blood concentrations, blood, at						ns, in mg per 100 cc i, at hours				Excreted in per cent of total dose at hours			
	g / kg	0 25	1	2	3	4	6	24	2	3	6	24	
Terrier I	0 1 0 2 0 5 1 0	14 09 18 16	28 33 70 72	41 72 94	3 8 3 2 4 5 7 2	28 16 42 52	1 2 1 4 2 2 4 1	01 03 00 02	20 4 19 4 23 2 24 3	50 1 39 2 29 8 49 7	62 7 49 6 64 4 59 4	76 0 67 4 71 2 73 9	
Greyhound 2	02	04 02	2 8 6 2	46	4 5 4 8	3 9 4 1	2 7 1 2	0 1 0 2	19 8 18 2	37 4 39 4	49 2 62 4	67 8 78 4	
Mongrel 3	05	1 0 0 2	4 2 4 7	3 9 4 9	2 6 6 2	1 9 5 3	2 1 4 0	0 4 0 2	17 2 24 6	28 4 33 2	42 6 47 6	66 4 75 2	
Terrier 1*	0 1 0 2	12 1 20 5	4 4 6 0	3 2 4 5	1 8 3 4	1 4 2 4	1 0 1 8	06 08	69 7 64 2	69 9 68 4	82 6 78 4	98 6 96 4	

TABLE VIII –

ABSORPTION AND EXCRETION OF SUILPHETRONE IN DOGS

tervals (Fig 6), suggested that absorption took place high in the alimentary tract. An experiment in which the drug was injected first into the large and then into the small intestine supports the view that absorption is largely confined to the small intestine (Fig 7)

Renal clearance in the rabbit

The essential preliminary to a study of the elimination of sulphetrone from the body is a knowledge of its mode of excretion by the kidney. In a study in the dog, Marshall, Kendall, and Cutting (1937) found the clearance of sulphanilamide to be 20 to 30 per cent of a simultaneously determined creatinine clearance. In an experiment in which a group of three rabbits was used the clearance of sulphetrone was 58 per cent that of creatinine, or two to three times as fast as sulph-

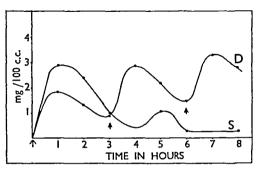


Fig 6—Blood concentration-time curves in a dog after sulphetrone by mouth S, a single dose of 0 2 g per kg in 60 per cent solution at first arrow D, a divided dose of 0 1 g per kg given at each arrow

anilamide The result was the same whether the plasma concentration of the drug was produced by prolonged administration for 40 days or by a single dose A typical estimate based on single doses is shown in Table IX Each ratio is a mean of six periods of one experiment, thus Rabbit X, 062, Y, 063, and Z, 05, giving a grand mean of 058

Renal clearance in the dog

Renal clearance for the dog has been calculated from data, some of which are given in the last

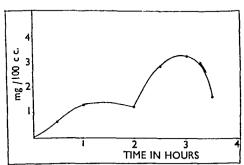


Fig 7—Blood concentration-time curves showing absorption from large and small intestines. Dog anaesthetized with pentobarbitone-sodium. Tube passed through oesophagus and pylorus into duodenum, and pylorus tied, a second tube passed through anus into the descending colon, the tube being tied at the anus to prevent leakage and the ileocaecal junction tied. Sulphetrone 0 I g per kg in 40 per cent solution introduced by the oral tube and washed out after two hours, 0 I g per kg then introduced into the duodenum.

^{*} Intravenously

TABLE IX SULPHETRONE AND CREATININE CLEARANCE IN THE RABBIT

The rabbit weighed 2.2 kg and received 1 g of creatinine and 3 g of sulphetrone dissolved in 25 c c of water. This quantity represented the third of three volumes of water given at 20 minute intervals

Period	Mins	Urine A c c /min	Crea mg /10 B plasma	tinine 00 c c C urine		netrone 00 c c C urine	Units	$\frac{\mathbf{C} \times \mathbf{A}}{\mathbf{B}}$ per min Sulphetrone	Ratio Sulphetrone Creatinine
1	27	0 57	9 2	350	41	102 0	2 15	1 4	0 65
2	37	0 39	11 6	133	75	56 6	4 45	2 94	0 66
3	30	0 25	12 0	160	85	59 2	3 35	1 75	0 52
4	23	0 83	11 2	265	89	46 7	19 5	4 3	0 22
5	60	0 34	10 1	240	40	171 5	8 2	6 44	0 78
6	60	0 37	8 3	270	89	232 4	11 5	9 6	0 98

experiment in Table VIII Six determinations gave a mean clearance $\pm S\,D$ of $85\,2\pm14\,3$ units, six sulphanilamide clearances on the same animal gave a mean clearance $\pm S\,D$ of $17\,9\pm3\,1$ units Accepting creatinine clearance as a rate of glomerular filtration it seems that some 40 per cent of sulphetrone is resorbed in the passage of glomerular filtrate along the tubules in the rabbit, while in the dog the quantity resorbed is very slight

Distribution of sulphetrone in tissue

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The penetration of sulphetrone into the tissues, with the exception of brain and cerebrospinal fluid, is rapid and complete. The distribution and concentration is the same whether the drug is given

TABLE X

The penetration of sulphetrone into various tissues in the rabbit and the dog, expressed as mg per 100 cc of fluid and mg per 100 g of tissue

Tissue	RABBIT (2 kg) 1% sulphe- trone in diet 10 weeks	RABBIT (175 kg) 100 mg sulphetrone per kg intravenously Estimates at 2 hrs	Doc (12 kg) 100 mg sulphetrone per kg intravenously Fstimates at 2 hrs
Blood Plasma Corpuscles C sternal fluid Bile Liver Kidney Spleen Lung Bone-marrow Striated muscle of thigh Ileum, empty erebral hemispheres Vitreous humour Fat	3 3 6 3 0 8 1 1 80 0 14 0 13 1 7 1 4 8 3 1 1 6 8 6 0 9 0 8	3 8 7 4 1 2 0 4 92 0 12 9 15 3 6 2 4.1 2 4 2 0 1 6 0 5 0 7 1 1	4 1 6 7 1 1 1 5 79 0 11 5 20 4 9 8 6 2 0 8 6 3 4 5 1 9 0 4 1 2

in the diet for prolonged periods or acutely by one intravenous dose (Table X)

The similar tissue concentrations in animals receiving drug by one intravenous dose, and in the diet for 10 weeks, point to the importance of renal clearance as one major controlling factor. In this connection the low concentration in plasma compared with liver and kidney, but not bone-marrow or lung, indicates that the liver also is functioning as an organ of concentration and excretion. The high concentration in bile supports this view. That neither the rate of excretion nor the route are the sole controlling factors may be deduced from Table XI, where the distribution in the tissues of the nephrectomized rabbit is given. In spite of striking increases in the plasma concentration

TABLE XI

The distribution of sulphetrone in the tissues of the nephrectomized rabbit at 2 hours after 100 mg per kg intravenously Kidneys were removed under pentobarbitone-sodium 30 mg per kg

Tissue	RABBIT (1 65 kg) mg/100 g or c c	RABBIT (1 60 kg) mg/100 g or c c	RABBIT (1 70 kg) mg/100 g or c c
I Hour Blood Plasma Corpuscles 2 Hours Blood Plasma Corpuscles Corpuscles Control fluid Corpuscles Control fluid Corpuscles Control fluid Corpuscles Control fluid Control	20 5 42 6 	22 9 53 8 12 4 43 0 6 2 trace 15 0 7 5 18 6 12 5 3 0 11 1 1 2	19 5 35 5 5 5 21 2 35 0 4 6 trace 170 0 11 9 8 1 13 8 8 1 2 6 8 5 0 9

(about five times) little change is reflected in the concentrations in the viscera. Biliary excretion is significantly high and concentration of drug in the ileum indicates that this, too, is an organ of excretion.

Other pharmacological properties

Examined by the usual pharmacological methods, sulphetrone is inert. Thus, a solution of 1 in 1,000 has no action on the isolated guineapig uterus or on the rabbit intestine *in vitro* at 37.5° C, or on the frog heart perfused through the vena cava. A cat anaesthetized with phenobarbitone-sodium (30 mg per kg) and given 0.2 g sulphetrone per kg intravenously showed no demonstrable effect on blood pressure, heart rate, respiratory frequency, or volume

EXPERIMENTAL THERAPY

Antibacterial activity

Our procedure for measuring antibacterial activity of sulphonamide-type compounds *in vitro* consists of observing the effect of exposing constant inocula of selected pathogens to successive dilutions of known concentrations of test and standard drug in media containing 10 per cent of blood Results of tests to determine the limiting inhibitory con-

TABLE XII

The limiting inhibitory concentrations of diaminodiphenylsulphone, sulphetrone, and promin against
different organisms in vitro

Organis			Diamino diphenyl sulphone	Sulphe trone	Promin
Str pyogenes D pneumoniae S aureus S salwarius """ E typhosa Sh sonnei Sh dysenteriae Sh paradysenteria E coli V comina Cl perfringens Cl septicum Cl novyi M avium "" "" "" "" "" "" "" "" "" "" "" "" ""	CN CN CN CN ar hom 844 271 1877	368 735 281 280 unis (H37)	20 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2	\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\	25 24 25 25 25 25 25 25 25 25 25 25 25 25 25

centrations of sulphetrone, promin, and diaminodiphenvisulphone in Wright's broth containing 10 per cent of blood against Gram-positive and Gramnegative organisms, and in Long's medium containing 10 per cent of blood against M ayum and M tuberculosis var hominis and var bovis, are summarized in Table XII Initial drug concentrations of 20 g per litre were made and diluted out in the medium to obtain a series of drug concentrations which decreased by multiples of two Drug solutions in the test medium were autoclaved. mixed with blood, and inoculated with 0.2 cc of a suitably diluted culture Size of inoculum and conditions of test were such as to demonstrate antibacterial activity under conditions which permitted turbid growth in the control tubes in less than 24 Initial bacterial viable counts in test mixtures were made by roll-tube counts

Reversal by p-aminobenzoic acid

Titrations in a semi-synthetic reinforced gelatine-hydrolysate medium containing added cystine and tryptophane, together with the essential growth factors, and inoculated with 1,300 viable organisms of S pyogenes N 10, enabled the concentration of p-aminobenzoic acid which just reversed the antibacterial action of sulphetrone to be found. The figure for eight estimates, derived from tenfold dilutions of p-aminobenzoic acid from 10 5 to 10 10 , was 1 5,000 \pm 500

Streptococcus and pneumococcus mouse infections

The chemotherapeutic activity of sulphetrone in mice against a β -haemolytic streptococcus infection (CN 10) and against a Type 1 pneumococcus infection (CN 33) was assessed in a survival test experiment in which groups of 30 mice receiving a diet containing 2 per cent of sulphetrone or of other sulphonamides were inoculated with a number of lethal doses of the test organisms diet intakes and blood concentrations were measured at 14 days after infection, at which time treatment was terminated Deaths were recorded daily and the cause of death ascertained by blood culture, survivors were observed for a further seven days The data from these experiments are recorded in Table XIII for the streptococcus-test in which sulphanilamide was used for comparison Sulphetrone is as good an antistreptococcal drug as sulphanilamide and in terms of a therapeutic factor F, obtained by dividing the mean free drug concentration in blood by the mean drug intake, it is more efficient Rather surprisingly, in view of the efficiency of diaminodiphenylsulphone, sulphe trone has no antipneumococcal activity

TABLE XIII

Three groups of 30 mice infected with 2 lethal doses (LD100) of Streptococcus pyogenes CN 10 All controls were dead within 48 hours and the survivors are shown after 21 days' treatment with 2 per cent drugs in the diet When the factor F, derived from groups of 25 mice on the 14th day, is considered, sulphetrone is the more efficient

Drug 2% in diet	Survi- vors	Mean drug intake mg	Mean free drug Blood conc in mg per 100 c c	F = blood conc drug intake	
Sulphetrone Sulphanil amide	25/30 27/30	69 2 ± 9 5 54 6 ± 3 8	$ \begin{array}{c} \hline 114 \pm 34 \\ 184 \pm 42 \end{array} $	$0.161 \pm 0.031 \\ 0.336 \pm 0.057$	

In vivo-in vitro antituberculous test

Before proceeding to a full-scale guinea-pig protection trial it is our custom to exact the following minimum therapeutic requirement from a potential antituberculous drug Guinea-pigs of 550-600 g are injected intraperitoneally with 2 g of the substance suspended in 10 per cent gum acacia and, after two hours or before if symptoms of acute toxicity are seen, they are anaesthetized, the thorax is opened and they are bled aseptically from the heart by Pasteur pipette By drawing the specimen while the heart still beats, no difficulty is experienced in obtaining more than 3 c c of blood, which is stored in a sterile citrated bottle The blood is diluted in serial increments with equal volumes of 05 cc of Long's agar contained in previously sterilized and stoppered Lambeth The tubes are sloped in the usual fashion tubes and sown with 001 cc of a uniform suspension of M tuberculosis containing 0.5 mg per c c The tubes are incubated at 375°C and inspected at intervals, the inhibition of growth is compared with the inhibition produced by diaminodiphenylsulphone tested in the same way at the same time Avian strains are read at six days, and bovine and human strains at 21 days A comparison of sulphetrone and diaminodiphenylsulphone is given in Table XIV

DISCUSSION

The preparation in these laboratories of diaminodiphenylsulphque and its characterization as a potent antibacterial and chemotherapeutic agent resulted in many attempts here and elsewhere to discover an equally potent but less toxic derivative With the knowledge of its chemotherapeutic antituberculous effect the search was intensified Sulphetrone was described in 1938 (Buttle et al., 1938),

TABLE XIV

Minimum effective drug blood concentrations in mg per 100 cc blood (from serially diluted blood of a guinea-pig given drug parenterally) which just inhibit strains of mycobacteria

Concentration of drug in guinea pig blood mg per 100 c c				Diamino- diphenyl- sulphone	Sulphe- trone		
wum			CN	- 5	281	0 14	0 22
<i>นบนนท</i>			CN	2	280	0 14	0 22
uberculosis	var bo	VIS	CN	8	358	75	110
			CN	8	868	19	55
uberculosis	var ho	minis	CN	8	344	75	110
		CI	N 2	71	(H37)	19	55
						19	110
"		,,					110
	wum wum uberculosis	wium wium uberculosis var bo nberculosis var ho	wium wium wherculosis var bovis nberculosis var hominis	with CN with CN whereulosis var bovis CN hereulosis var hominis CN ,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	with CN 2 with CN 2 with CN 2 whereulosis var bovis CN 3 whereulosis var hominis CN 8 y, , , , , , CN 271 y, , , , , , , CN 18	wium CN 281 wium CN 280 whereulosis var bovis CN 858 con 868 whereulosis var hominis CN 844 con 1877 con 1877 con 1877 con 1878	wium CN 281 0 14 wium CN 280 0 14 with CN 280 0 14 whereulosis var bovis CN 858 75 CN 868 19 whereulosis var hominis CN 844 75 cn 1877 19 cn 1877 19 cn 1877 75

when its chemotherapeutic antistreptococcal effect was appreciated, but it was not until 1941 that its chemotherapeutic antituberculous action was first realized While sulphetrone was still under test, preliminary reports of promin (Feldman, Hinshaw, and Moses, 1941), the first diaminodiphenylsulphone derivative to be used in man for the treatment of tuberculosis, appeared This drug, and the derivatives which have appeared since, diasone and promizole, are very toxic drugs when administered orally and may be given only for short intermittent periods The fact that sulphetrone proved to be so relatively non-toxic, coupled with the hopes that clinical trials of this kind have always engendered, led us to the conclusion that all publication should be suspended until a mature appreciation could be given, with the publication of clinical reports this can now be done

The antibacterial activity of sulphetrone approaches closely that of its parent substance, diaminodiphenylsulphone, and suggests that it may prove equally effective in the treatment of experimental tuberculosis in the laboratory animal

The acute toxicity of sulphetrone when given by mouth is so slight that it cannot with certainty be determined, but the studies are complicated by the soluble nature of the drug which necessitated the administration of grossly hypertonic solutions. On the basis of blood concentrations sulphetrone would seem to be many times less acutely toxic than sulphanilamide. This is in marked contrast to the studies on acute toxicity undertaken with promin, diasone, and promizole

The fact that sulphetrone is not acetylated, or conjugated in any other way, throws an interesting light on this lack of toxicity. The implication of this finding is that the drug is not hydrolysed to diaminodiphenylsulphone, an inference which is implicit in the lack of acute toxic symptoms observed in the dog with a very high blood level of

sulphetrone Since in its suggested use, the problem of dosage is to maintain an effective consistent level in blood and other tissue for very long periods of time, we have been interested in the chronic toxicity of long-maintained blood levels

Chronic toxicity experiments indicate that very large doses can be given by mouth to mice and dogs without producing symptoms or pathological changes in the tissue With similar doses in rabbits, very interesting haematological changes are First, there is a small but continuous haemolytic anaemia which is marked by a concurrent reticulocytosis Secondly there is a progressive hypochromic anaemia of iron-lack anaemia is due to competition for alimentary iron by sulphetrone which forms an insoluble iron salt, which is not absorbed It may be prevented or cured by the administration of iron, parenterally or orally

The third anaemia which is seen is slower to appear but is then precipitous, it is of nutritional origin and may be prevented or cured by the administration of dried yeast. Its probable cause is the limitation and alteration of the bacterial flora of the gut by the concentration of sulphetrone which is present

A specific toxic effect which sulphetrone shares with all sulphonamides is the property of causing hyperaemia and hyperplasia of the thyroid gland after courses of treatment similar to those in therapeutic use The hyperplasia is believed to result from hypothyroidism owing to failure to synthesize thyroid hormone

Experiments in groups of rats maintained on a diet containing 1 per cent of the drugs for 17 days showed sulphetrone to be the least, and sulphaguanidine the most, toxic, while sulphadiazine was intermediate in this respect

In a further study of chronic toxicity, it was observed that 1 per cent concentrations of drug in the diet of groups of rats retarded normal growth for an initial period of 14 days, but thereafter increases in weight followed the curve of normal increments during a 78-day period of observation After this long period of treatment, the increases in the weight of the thyroids were not significantly greater than those in the control groups

The lack of acute toxicity and the low chronic toxicity, with complete freedom from the toxic sequelae seen after administration of diaminodiphenylsulphone, make it clear that sulphetrone is not degraded to diaminodiphenylsulphone in the body. However, the possible hydrolysis of one or more of the four sulphonated side chains could not be overlooked and alkali reserve experiments were

made to study this point Simple hydrolysis resulting in the liberation of one or more molecules of sodium acid sulphate seemed the most likely event Experiments in rabbits receiving oral and parenteral sulphetrone and experiments in dogs with the drug administered parenterally showed, however, that alkali was liberated in the blood stream, resulting in consistent increases in alkali reserve Comparison with parenteral sodium bicarbonate makes it possible to say that the increase corresponds to one molecule of sodium hydroxide being liberated from each molecule of sulphetrone A theoretical equation, involving the intervention of one molecule of water for the condensation of two molecules of sulphetrone through an -SO₂-O-SO₂linkage with the liberation of two molecules of sodium hydroxide, may be proposed 2R SO₂Na+ H₂O=R SO₂OSO₂R+2NaOH Experimental evidence in man points to the existence of a complex condensation product of this kind When chronic tests were made on groups of rabbits on a "crossover test" basis, no significant increases in alkali reserve were recorded eighteen hours after the last dose, at which time the average blood levels were 70 mg per 100 cc It seems that under these conditions the normal animal is able to re-establish an equilibrium in its plasma alkali balance

Reviewing all the evidence of toxicity in the experiments described, it seems safe to say that when given by mouth not only is sulphetrone the least toxic of the sulphones, but it is also less toxic than any of the sulphonamide drugs

Experiments in mice and dogs in which single small or large doses are given show that after a certain point is reached large increases in dose do not result in either a higher blood concentration or increased absorption This is not an unusual finding with sulphonamide drugs, but it seems to occur with sulphetrone at a lower level of dosage The rabbit is anomalous in that increase in dosage increases the absorption and blood concentration of sulphetrone The same total amount of drug given in divided doses at intervals of a few hours results in a higher blood concentration than when The explanation given as a single dose (dog) seems to be that this very soluble drug is slowly absorbed, and mainly from the small intestine In addition, the rate of excretion of sulphetrone by the kidney is very fast. In rabbits the clearance of sulphetrone was 58 per cent that of creatinine or two to three times as fast as sulphanilamide, while the clearance in the dog was five times as fast as sulphanilamide If creatinine clearance is accepted as a measure of the rate of glomerular filtration it seems that in the rabbit some 40 per

cent of sulphetrone is resorbed in the passage of glomerular filtrate along the tubules while in the dog the quantity resorbed is very slight. Some part, therefore, of the higher blood concentrations found in the rabbit is contributed by slower clearance

Sulphetrone penetrates all tissues with extreme rapidity, with the exception of brain, but it enters the cerebrospinal fluid rather more slowly than do other sulphonamides

The tissues of animals receiving the drug intravenously show similar concentrations to those receiving the drug in the diet for 10 weeks normal animal concentration of drug in the liver. kidney, and spleen is always greater than in the The relation between absorbed drug, drug in transport, and drug in kidney clearance does not appear to be a simple one, and there appears to be an additional factor limiting drug concentrations in tissue such as liver, spleen, and The plasma levels in the nephrectomized rabbit make an interesting comment on this observation since they are many times the plasma level in the normal animal and many times the level in the other tissues In both normal and nephrectomized animals the concentrations in bile are very high, in the normal they are some 12 times the plasma levels and may be considered as drug in transport, thus making a significant contribution to the clearance of the drug. This view receives support from the fact of the slight decrease in the plasma concentrations in the nephrectomized rabbits between the first and second hours, the average fall in plasma concentration is some 5 mg per 100 cc which, in a rabbit of 17 kg, containing by our records 200 cc of plasma, corresponds to a total in bile of 10 mg of sulphetrone The recorded bile flow of these anaesthetized rabbits averages 10 cc per hour, which is equivalent to a sulphetrone loss of 15 mg may therefore be argued that in the nephrectomized animal sulphetrone reaches equilibrium within one hour, and the subsequent fall in plasma concentration is due to biliary excretion and not to metabolic destruction

Sulphetrone has no action on smooth muscle, heart, blood pressure, or respiration in concentrations usual in pharmacological studies

In considering the data provided by the antibacterial activity of sulphetrone in vitro little significance is to be attached to the absolute values recorded for effective inhibitory concentrations. On the other hand, the comparative values for diaminodiphenylsulphone, sulphetrone, and promin are of high significance. In the many comparisons of this kind which have been made in these laboratories the inhibitory concentrations of sulphetrone against mycobacterial strains have always been found to be lower than those observed with promin, and this although, weight for weight, promin contains more diaminodiphenylsulphone. The implication is that the potential cinnamylidene linkages of sulphetrone contribute a significant antibacterial function. No support for this claim can be derived from reversal experiments with paraminobenzoic acid, which reverses the antibacterial rates of sulphetrone at a concentration of 1 5,000

It is surprising, in view of the known efficiency of diaminodiphenylsulphone in pneumococcus infections in mice, that sulphetrone is inactive, however, in streptococcus infections in mice, sulphetrone is more effective than sulphanilamide

We have introduced the *in vivo-in vitro* antituberculous test in order to bridge the gap in the difficult transition between antituberculous *in vitro* tests and animal protection experiments with strains of mycobacteria. Whenever the drug can be estimated, and it is seldom that it cannot, either chemically or microbiologically, it is possible to express the inhibition of the organism in a quantitative fashion in terms of a standard such as diaminodiphenylsulphone. When the drug cannot be estimated, the method depends on assessing the inhibition of the organisms produced by the quantity of drug existing in the blood after a dose sufficient to produce toxic signs in the animal, and therefore at an optimum level

An assessment of the efficiency of sulphetrone in the treatment of experimental infections with strains of M tuberculosis in the guinea-pig is the subject of a separate communication. A study has also been made of the toxicity of the drug when used in man Certain advantages and disadvantages of sulphetrone as a potential chemotherapeutic agent in man may be deduced from the present study Absorption is slow and excretion is so rapid that limitation of fluids may be essential to maintain an adequate blood level ever, the fact that the drug is not acetylated means freedom from kidney complications and freedom from the toxic manifestations associated with the use of diaminodiphenylsulphone

The record of clinical trials concurrently appearing in the press should enable a preliminary assessment of the toxicity and therapeutic status of the drug to be made

SUMMARY

1 The chemical and physical properties of 4 4'bis(γ-phenyl-n-propylamino) diphenylsulphonetetrasodium sulphonate, given the trade name of "sulphetrone," together with its absorptiometric estimation in body fluids, are described

- 2 The acute toxicity of the drug has been investigated in mice and dogs and its chronic toxicity in rabbits
- 3 A haemolytic anaemia, an anaemia of iron lack, and an anaemia of nutritional origin arising in the course of chronic sulphetrone administration in rabbits have been investigated and the conditions for their successful treatment indicated
- 4 The specific goitrogenic effect, shared by all sulphonamide drugs, has been investigated and shown to be of slight degree
- 5 In acute experiments sulphetrone given orally or parenterally raises the alkali reserve of the plasma in the rabbit and the dog. The probable mechanism is discussed. When the drug is administered over a period of time equilibria are established.
- 6 On the basis of these experiments, together with studies of the influence of the drug on normal growth of rats, it is concluded that sulphetrone is virtually non-toxic in acute experiments, and has low toxicity in prolonged ones
- 7 Although exceedingly soluble in water, sulphetrone is slowly absorbed from the intestinal tract, most from the small intestine, little from the large
- 8 The drug is not conjugated in the experimental animal or in man
- 9 Sulphetrone penetrates all tissues, with the exception of brain, very rapidly, and is present in them to about the same concentration as in blood. It passes into the cerebrospinal fluid much more slowly than the simple sulphonamides do
- 10 Sulphetrone has no action on smooth muscle, heart, blood pressure, or respiration
- 11 Antibacterial *in vitro* studies, in the presence of blood, show sulphetrone to approach the efficiency of its parent substance diaminodiphenyl-sulphone against two strains of *M avium*, two

strains of *M* tuberculosis var bovis, and three strains of *M* tuberculosis var hominis

- 12 The drug is rather more effective against β -haemolytic streptococcus infection in mice than sulphanilamide, but, unlike the parent substance, diaminodiphenylsulphone, it is ineffective against a pneumococcus infection
- 13 In an *in vivo-in vitro* test, blood from a guinea-pig previously given parenteral sulphetrone inhibits, *in vitro*, strains of virulent mycobacteria
- 14 The pharmacological properties of sulphetrone suggest that it may prove effective in the treatment of experimental tuberculosis in the laboratory animal, and that its administration to man, in large doses for protracted periods, is a practical possibility

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THE TREATMENT OF EXPERIMENTAL TUBERCULOSIS WITH SULPHETRONE*

BY

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"Sulphetrone" is the registered name for bis(γ -phenyl-n-propylamino) - di - phenylsulphonetetrasodium sulphonate, the pharmacology and chemotherapy of which have been recently reported (Brownlee, Green, and Woodbine, 1948) Sulphetrone has a low toxicity and an antituberculous efficiency approaching that of its parent compound, diaminodiphenylsulphone, it is also curative in infections due to β -haemolytic streptococci Moreover it satisfies the requirements of an in vivo-in vitro comparison with diaminodiphenylsulphone, the concentrations of these substances in guinea-pig blood after massive intraperitoneal injections are capable of inhibiting the growth of virulent strains of Mycobacterium tuberculosis The present report is concerned with the treatment of experimental tuberculosis of both human and bovine types in the guinea-pig

METHODS AND MATERIALS

Animal management

In carrying out screening tests of chemotherapeutic agents against M tuberculosis there are two essentials protection of staff from the risks of infection, and prevention of intercurrent infection in the animals under experiment, these can be achieved only by continuous and vigilant cleanliness on the part of a co-operative and dependable staff of helpers It is an advantage to adopt a rigid daily routine which is on record available to and approved by all concerned The animal room is 8 ft by 30 ft, with transom windows along the long side and with a single entrance set at the opposite end of the long side. In order to assist ventilation it consists of a frame filled with fine copper gauze, with which the windows are The door opens into a vestibule also screened

'lock" enclosed by frame-work and door of wire gauze. This arrangement reduces the potential passage of all possible vectors and serves in addition to emphasize the "isolation" nature of the work. Walls are of breeze-block and plywood, filled, and glossy enamelled, and the ceiling is of "cellotex," and is similarly treated. Switches, cable, and light fittings are waterproof. The floor, of concrete, is treated with spindle-oil at two-monthly intervals, and is scrubbed weekly. The whole is washed down at two-monthly intervals. Temperature is held approximately at 20° C and is controlled by hand-controlled steam-heated radiators and an extractor fan

Guinea-pigs are housed six to a galvanized-wire cage fitting in a sheet-metal tray The floor space per anımal ıs 48 sq ın The animals are identified by a combination code of natural colouring and artificially applied dve White-enamelled metal hookon labels have been found the most satisfactory after trials of many other types and of other devices Food containers of tin-plate are non-spill and consist of an annular moat of 8-in diameter with a central hollow projecting pillar to prevent soiling, and hold 250 g of dry diet. Glazed non-spill water-pots holding 120 cc of water, are refilled three times daily Absorbent litter is large wood shavings which scatter less than does sawdust. Weights are recorded weekly with a spring-pan balance weighing Changing to clean, previously autoclaved cages is done daily, except Sunday Soiled cages, together with feeding-troughs and water-pots, are steamed before cleaning, and for this and similar purposes a double-door autoclave connects the animal room to the cage-cleaning room

Guinea-pigs

In recent years guinea-pigs bred and reared on our own farm have been used. This has created a further problem since these animals, in addition to being much less prone to intercurrent infection, are more resistant to artificially induced tubercle infections than are the guinea-pigs of commerce

^{*}The development of sulphetrone is part of the programme of work on antituberculous compounds carried out by the Therapeutic Research Corporation of Great Britain Limited The product was made and will be issued by the Wellcome Foundation Ltd

Diet

The dry, finely ground diet is based on that of Coward (1937) adapted to guinea-pig requirements. It is made up as follows

Tubercle Diet No 2	
Yellow maize, whole, ground fine	700
Wheat, whole, ground fine	250
Milk, winter, dried (Glaxo)	50
Casein (BDH)	90
Yeast, dried (Pharmaco-Chem Prod)	150
Salt mixture	12

The salt mixture has the following composition manganese sulphate 01, potassium iodide 10, ferric citrate 10, sodium chloride 600, and calcium carbonate 1,0000. The ingredients are mixed mechanically, and drug, when added, is mixed separately. The rate of consumption of this diet is 30-40 g. daily in guinea-pigs weighing 450-500 g., and their diet is supplemented with 100 g of greenstuff.

Mycobacterium tuberculosis strains

The human strain CN 271 (H 37) is the virulent Saranac strain obtained from the Trudeau Sanatorium, NY, in 1931 and maintained on synthetic fluid medium by implant transfer at 21 days (Steenken and Gardner, 1946) In our hands this strain is moderately virulent. The bovine strain CN 858 (AN 5 Weybridge) has proved to be apparently stable, by virulence titration, since its acquisition in 1943. It is highly virulent. In recent years both strains have been kept by Mr H Proom in a dried form which may prove to be the method of choice for maintaining virulence.

Grown on a solid egg-medium (Petragnani) for 14 days, suspensions are prepared by mechanically shaking with distilled water in a stainless steel bottle with steel balls and adjusted to 0.5 mg per c c by reference to a previously standardized vaccine

The estimation of sulphetrone in body fluids

The estimation of sulphetrone in body fluids has been described (Brownlee et al 1948) and is based upon diazotization and coupling to N(1-naphthyl)-ethylenediamine hydrochloride (Bratton and Marshall, 1939) The essential differences he in the dilution of 1 in 15 and the concentration of acid, both of which are critical Blood is obtained from the ear of the guinea-pig, from which, with patience and practice, up to 0.5 c c may be drawn into a clean heparinized capillary tube

In Fig 1 are given the blood-concentration time curves obtained with 1, 2, and 4 per cent of sulphetrone in the powder diet, and also the curve corresponding to a 2 per cent diet when greenstuff is fed in addition

FIRST EXPERIMENT Bovine Strain CN 858

Forty young adult guinea-pigs of mixed sexes, weighing 450-500 g, were inoculated deeply into the right thigh muscles with 0.25 mg of a fourteenday-old sub-culture of bovine tubercle bacillus,

CN 858 The course of the infection was rapid, so that by the end of the second week glands were much enlarged and several animals had open abscesses. On the fifteenth day the animals were divided into two groups each of 20 animals, one to serve as control and the other to be the drugtreated group. Thereafter each animal in the latter group received in its food an estimated dose of 600 mg of sulphetrone for the duration of the experiment. The blood sulphetrone concentration corresponding to this daily dose gave a mean value of 5 mg ± 4 SE for weekly estimates during the course of the experiment.

RESULTS

Survival time—A record of the weights and survival times of the animals is given in Fig 2, from which it will be seen that the first animal in the untreated group died at 35 days and the last at 77 days after infection. About three-quarters of the animals were dead by 50 days. At a time when 50 per cent of animals in the control group were dead, 25 per cent were dead in the treated group, when all controls were dead, 25 per cent of the treated animals were still alive. The last animal in the drug treated group died 237 days after infection and 160 days after the last of the untreated animals had died. The average survival time after infection was 45 days in the untreated group and 77 in the treated group.

Necropsy studies - Post mortem examination showed gross tuberculosis in 19 out of 20 animals in the untreated group, and one animal with severe tuberculosis The nature of the infiltration was confirmed by histological examination and the organism was recovered by culture on solid eggmedium (Petragnani) In the drug-treated group of 20 animals, 9 showed gross, 5 severe, and 5 moderate tuberculosis, in one the only sign was a minute abscess at the site of injection from which The nature of the the organism was recovered disease was confirmed by histological examination and the organism was recovered in 19 out of 20 anımals

The extent of the disease in the animals of both groups is shown pictorially in Fig 3

SECOND EXPERIMENT Human Strain CN 271
(H 37)

Forty-five young adult guinea-pigs of mixed sex, weighing 450-500 g were inoculated intramuscularly deep into the right thigh with 0.25 mg of a 21-day-old culture of CN 271 (H 37) Fourteen days after infection the animals were divided

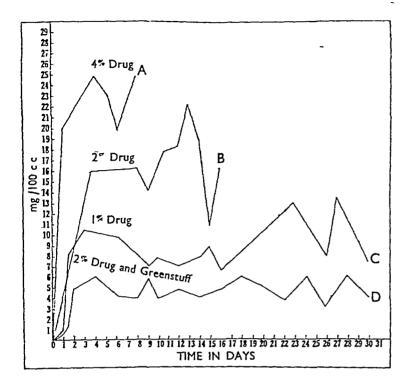
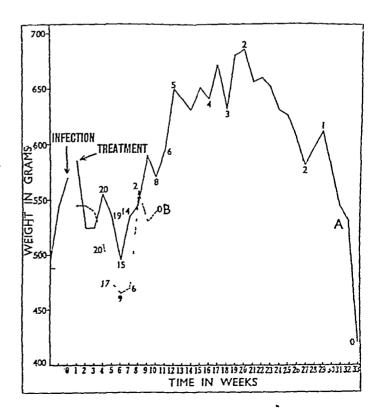


FIG 1—Blood concentration-time curves after administration of sulphetrone in the diet. Each curve is the average of estimates on a group of five guinea-pigs weighing 450-500 g. A, sulphetrone 4 per cent, equivalent to 14 g. daily, B, 2 per cent, or 0.7 g. daily, and C, 1 per cent, or 0.35 g. daily. In addition to 2-per cent drug in diet group D received 100 g. of greenstuff daily

FIG 2.—Weights and survival times of guinea-pigs after intramuscular infection with a bovine strain of tubercle bacillus A, treated with sulphetrone, B, untreated The figures on the graphs refer to survivors



into two groups, one of 21 animals as a control, and the second of 24 animals as the drug-treated group, the latter group received 2 per cent of sulphetrone in the diet. The blood sulphetrone concentration corresponding to this daily dose, derived from weekly estimates made during the course of the experiment, was $48 \text{ mg} \pm 28 \text{ SE}$

RESULTS

Survival time - There was a considerable difference in the survival times of the untreated and treated groups (Fig 4) When the last of the controls had died at 154 days only 25 per cent of the group that had received sulphetrone had died only one guinea-pig of the untreated group was the cause of death possibly not due to tuberculosis At the 154th day the 18 survivors in the drugtreated group were divided into two groups, one called drug-continued group, which continued to receive drug until the experiment terminated at 61 weeks when the last survivor was killed, and the other a drug-discontinued group from which drug was withdrawn These two groups afford an interesting parallel Within the drug-discontinued group deaths occurred regularly until the last animal died on the 280th day, or 126 days after the last animal in the control group had died. At this stage 50 per cent (4/9) of the animals in the drug-continued group were still alive

Necropsy studies — The extent of the disease in both groups of animals is shown pictorially in Post-mortem examination showed gross tuberculosis in 13 out of 21 animals in the control group, five animals had severe tuberculosis, two moderate, and one in which the infection was minimal The gall bladder and the small intestines of this animal showed the appearance characteristic of salmonella infection and there seems little doubt that this was the cause of death. The recovered organism was identified by Mr Proom as Salmonella bovis-morbificans The extent of the tuberculosis in all the drug-treated animals was very much less than in the untreated group the same period of 154 days in which all the controls died, three drug-treated guinea-pigs died with gross tuberculosis, one with severe and two with moderate lesions Within the drug-discontinued group of nine animals, three showed gross tuberculosis, three severe, and three moderate Within the drug-continued group two had gross, and four moderate tuberculosis, and in one animal the disease was minimal The organism was recovered in all cases by culture methods

Histology —Histological studies confirmed the extent and nature of the disease Not only was

its distribution and extent in the treated group very much less but the numbers of acid-fast organisms were noticeably less. In addition there was much evidence of healing in lung, liver, spleen, and glands The numerous regressive lesions with fibroblastic changes, many of them calcified, were impressive (Figs 6 and 7) This change was observed also in lymph nodes in a number of animals There was more tuberculosis of a progressive nature in the drug-discontinued group than in the drug-continued group, but here again there was impressive microscopic evidence of healing picture was that of a resurgence of tuberculosis which had been repressed by the drug, in the lungs this had the appearance of miliary tuberculosis (Fig 7)

DISCUSSION

The design of an animal experiment calculated to assess the therapeutic worth of a potential antituberculous agent for man is a critical exercise fraught with difficulty Tuberculosis in the experimental animal is a very different disease from tuberculosis in man in whom it is frequently characterized by an insidious onset, a chronic course, and a liability to relapse Yet, the ultimate goal may be simply stated as the elimination of virulent tubercle bacilli, a point on which there is universal agreement (Feldman, Hinshaw, and Mann, 1944) The absence, to date, of a substance with this ideal requirement has dictated the evolution of other standards of comparison have been the comparison between survival-times of drug-treated and untreated groups of animals, a comparison of the nature and extent of tuberculous lesions in the organs of predilection, and a comparison between pieces of liver obtained by biopsy and at the final post-mortem examination Ideally, a standard of comparison such as diaminodiphenylsulphone is included in the test. In a series of classical reports, Feldman and his collaborators (1940, 1942, 1943, 1944, 1945) have evaluated the status of promin and diasone, derivatives of diaminodiphenylsulphone, and promizole Probably by reason of the exploratory nature of their first experiments these workers adopted the use of what may be called a minimal lethal infection, using the virulent Saranac strain of H 37, and examined the treated animals at the time when the last control animal had died This kind of experiment presents an effective anti-tuberculous agent in its most favourable light. Nevertheless, this is an entirely satisfactory practice when comparisons are made, especially when taken together with the biopsy control technique, and it has enabled Feldman and

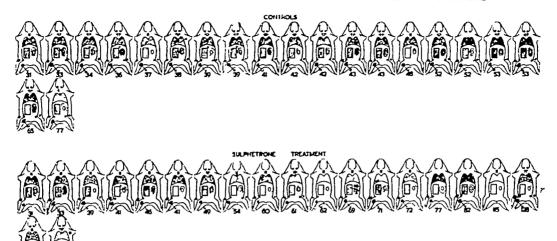


Fig 3—First experiment Guinea-pigs infected with 0.25 mg of the virulent bovine strain of tubercle bacilli CN 858 A pictorial record of the gross amount and distribution of tuberculosis seen at death in untreated and sulphetrone-treated animals. The number beneath an animal shows the days of life after infection Although observed until death from tuberculosis, there is less gross disease in the treated group

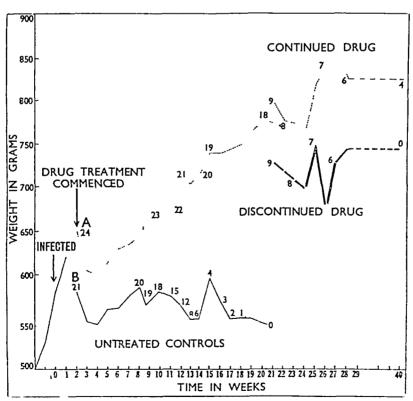


Fig 4—Second experiment Weights and survival times of guinea-pigs after intramuscular infection with a human strain of tubercle bacillus A, treated with sulphetrone, B, untreated When all controls had died, the 18 survivors in group A were subdivided to give a drug-continued group and a drug-discontinued group. The figures on the graphs refer to survivors

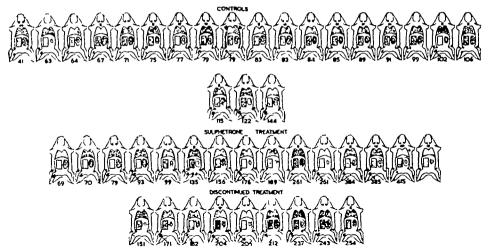


Fig 5—Second experiment Guinea-pigs infected with 0.25 mg of the virulent human strain of tubercle bacilli CN 271 (H 37) A pictorial record of the gross amount and distribution of tuberculosis seen at death in untreated and treated animals. The number beneath an animal shows the days of life after infection. Although observed until death from tuberculosis, there is less gross disease in the treated group.

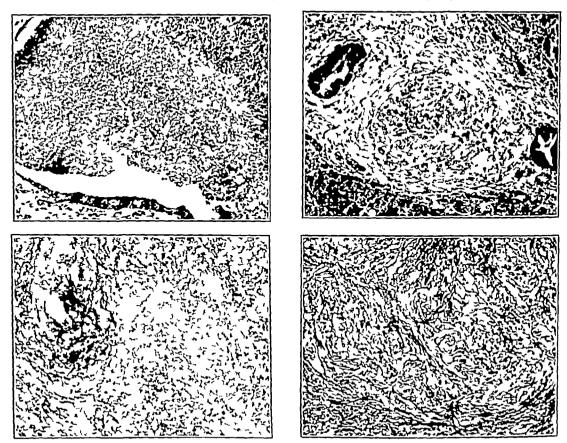


Fig 6—Second experiment H 37 human strain (Top left) Area of progressive destructive tuberculosis in the liver of an untreated guinea-pig which died 143 days after infection with tubercle bacilli (× 66) (Top right) Fibrotic healing tubercles in the liver of a guinea-pig treated for 384 days with sulphetrone (66) (Bottom left) Progressive destructive tuberculous area in an inguinal lymph node of an untreated infected guinea-pig which died 143 days after infection (× 123) (Bottom right) Non-progressive, markedly fibrotic involution in an inguinal lymph-node of a guinea-pig treated for 384 days with sulphetrone

his collaborators to evaluate the status of chemotherapeutic antituberculous drugs. It is their opinion, with which we agree, that the sulphone drugs are not capable of eliminating virulent organisms from the organs of predilection in the experimental animal

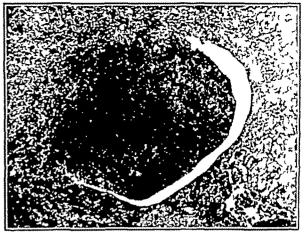
In making this first report of the antituberculous activity of sulphetrone it seemed proper to record tests which presented the drug in its least favourable light

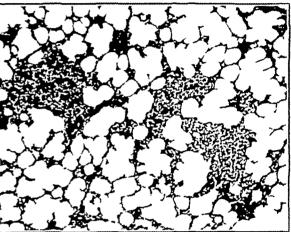
In the experiments reported here, the amounts of the virulent strains inoculated were large, and in addition to an experiment with a human strain, a test with a bovine strain is reported. Also, the animals were observed until death. In another place comparative tests with other drugs will be reported

Sulphetrone is a derivative of diaminodiphenylsulphone of low, acute and chronic toxicity As will be reported elsewhere, its lack of toxicity is such that doses equivalent to those given to guineapigs and sufficient to give blood concentrations of 5 to 75 mg per 100 cc have been administered to man for continuous periods of 12 or more months In this respect it differs from promin and diasone whose toxicity is such that they may be given for only short and intermittent periods. Since others have considered that the therapeutic action of promin and diasone may be due to some extent to their degradation to diaminodiphenylsulphone, it is interesting that with sulphetrone the evidence, both chemical and pharmacological, is that this breakdown does not occur

In the first test the experimental conditions, both in respect to the amount of the virulent infecting organism and to its bovine origin, were severe Nevertheless, the group of infected guinea-pigs receiving 2 per cent of sulphetrone in their diet showed a significant prolongation of survival-time For example, at a time when half of the control animals were dead, only one-quarter of the treated group were dead, when all control animals were dead, one-quarter of the treated group were still alive Expressed in terms of average survival days the figure for the treated group was 77 days, and for the untreated group 45 days

The inoculum used in the second experiment was the large injection of 0.25 mg of the human virulent Saranac strain H 37. In this instance the protection given by drug treatment was large. For example, when the last of the control animals had died, three-quarters of the treated animals were still alive, and as Fig. 4 shows, were in very good physical condition. In contrast, the animals from the control group showed gross tuberculosis, and histological examination revealed the presence of







strain (Top) Severe necrotizing progressive tuberculosis in the lungs of an untreated guinea-pig which died on the 143rd day after infection with tubercle bacilli (×123) (Centre) Discrete tuberculous nodules consisting of coalescent voung miliary tubercles from the lungs of a guinea-pig treated for 154 days with sulphetrone and then treatment discontinued Died on the 204th day (×123) (Bottom) Calcified fibrotic nodule in the lungs of a guinea-pig treated with sulphetrone until death on the 189th day (×123)

numerous tubercle bacıllı By dividing the survivors into two groups, one in which drug was continued and one in which drug was withdrawn, a very significant observation was made. The order of protection conferred by the drug was continued in one group and withheld in the other Expressed in figures, it is shown that at the time when all members of the drug-discontinued group were dead, one-half of the drug-continued group were still alive Throughout the entire drug-treated group macroscopic evidence obtained at necropsy showed very much less tuberculosis than in the untreated group This was confirmed by histological examination and amplified by the observation that acidfast organisms were very much less in number than in the untreated group, where they were numerous The most significant histological evidence was the repeated finding of healed tuberculous lesions in spleen, liver, lungs, and lymph nodes, often they were calcified, particularly in lungs and lymph nodes

In both experiments there is evidence of a therapeutic effect present only in the groups treated with sulphetrone. With both the bovine and the human strains the results suggest that sulphetrone exerts a retarding effect on the progressive nature of established experimental tuberculosis in the guinea-pig. It should be remembered that the conditions of the tests gave the infection the optimum advantage. In addition, in the experiment using the human strain, there was impressive histological evidence of a reversal from progressively destructive disease to one in which the morbid process was retarded, there was also evidence of resolution and calcification.

The therapeutic effect is shown most markedly by the difference between percentage survivors in the treated and untreated groups, but it is evident that sulphetrone, in common with other similar chemotherapeutic agents, is incapable of eliminating the causative organism

SUMMARY

1 The chemotherapeutic value of 44'-bis(γ -phenyl - n - propylamino) - diphenylsulphone tetrasodium sulphonate given the trade name of sulphetrone, in treating experimental tuberculosis in guinea-pigs is described

- 2 When sulphetrone was added to the diet in 2 per cent, average blood concentrations of 5 mg per 100 cc were found throughout the experiments. The average amount of drug eaten was 0 6 g daily
- 3 In an experiment in which two groups of 20 animals were infected with a heavy inoculum of the virulent bovine strain of tubercle bacilli CN 858, the survival time of the drug-treated group was prolonged. The average survival time was 45 days in the untreated group and 77 days in the treated group
- 4 In a second experiment in which the infection was a heavy inoculum of the human virulent H 37 strain (CN 271), the treated group of 24 animals survived considerably longer than the untreated group of 21 animals
- 5 There was a resurgence of tuberculosis of a miliary kind in a group of guinea-pigs in which drug treatment was stopped
- 6 In the drug-treated animals which died there was impressive histological evidence of healed tubercles
- 7 Under the severe conditions of the experiments, sulphetrone was capable of exerting a sup pressive effect on the progressive nature of the experimental infections. Sulphetrone is bacteriostatic and, in common with other derivatives of diaminodiphenylsulphone, is not capable of eliminating the infective organism from animal tissue.

We are indebted to Dr David Trevan for the histo logical preparations and for assistance with the pathology He is also responsible for the photomicrographs

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THE CHEMOTHERAPEUTIC ACTION OF STREPTOMYCIN, SULPHETRONE, AND PROMIN IN EXPERIMENTAL TUBERCULOSIS*

BY

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The accumulated evidence from both experimental chemotherapeutic and clinical sources makes it possible to say that while streptomycin approaches closer than any other known chemotherapeutic agent to the concept of a completely effective antituberculous drug, it fails to eliminate unconditionally the causative organism nificance in this connection is the clinical evidence presented elsewhere (Madigan, Swift, and Brownlee, 1947b) that the antibiotic appears to be most effective in rapid progressive disease, and least effective in chronic disease The metabolism of the organism seems to be implicated in these effects, since in young dividing cells streptomycin is markedly effective, while it is ineffective against old resting cells It appears to be significant that when smaller infections of tubercle bacilli are used (01 mg H 37, Feldman, Hinshaw, and Mann, 1945) the results are better than when larger infections are used (10 mg A 27, Smith and McClosky, 1945)

From theoretical considerations of what is known of the mode of action of diaminodiphenylsulphone drugs, it is not surprising that sulphones and streptomycin show a synergism of action The synergism between streptomycin and promin demonstrated by Smith and McClosky (1945), and between streptomycin and diasone by Callomon, Kolmer, Rule, and Paul (1946), is of a high order and, in view of the relative failure of streptomycin by itself, suggests the possibility of combined clinical therapy Sulphetrone, the pharmacology and chemotherapy of which has been recently described (Brownlee, Green, and Woodbine, 1948), is a diaminodiphenylsulphone derivative of low chronic toxicity which is effective in experimental tuberculosis and which may be used to maintain effective blood concentrations in man for continuous periods of 12 or more months (Brownlee and Kennedy, 1948)

The present report describes a comparison between the chemotherapeutic antituberculous activity of streptomycin, sulphetrone, promin, and combined streptomycin and sulphetrone

METHODS AND MATERIALS

A recent account (Brownlee and Kennedy, 1948) has appeared of the methods adopted in the management of guinea-pig tests. The same report should be consulted for the composition of the dry powdered Tubercle Diet No 2 which constitutes the basic ration

Mycobacterium tuberculosis strains

The strain CN 844 used in the present tests is a virulent human strain isolated in 1942 from a case of tuberculous adenitis, and maintained on synthetic fluid medium by implant transfer at 21 days. This virulent strain gives rise to a more chronic type of disease than the virulent Saranac strain CN 271 (H 37) previously used. This change was dictated by a temporary loss of virulence in the H 37 strain, a feature discussed by Steenken and Gardner (1946), who make suggestions for avoiding this complication.

Streptomycin

The sample of streptomycin sulphate used in this test was drawn from WF Batch 17 and is of 33 per cent purity. The chemotherapy and pharmacology of this batch together with the method of assay have been described (Madigan et al, 1947a). The potency of streptomycin is stated in terms of weight of pure streptomycin base $C_{21}H_{37}N_{7}O_{42}$ (mol wt.579) and is derived by assay with a working standard of streptomycin sulphate ($1\frac{1}{2}$ H SO₄, mol wt 726) of known potency, it follows that streptomycin sulphate contains 798 per cent of base

Sulphetrone

'Sulphetrone" is the registered name of the Wellcome Foundation Ltd for 4 4'-bis(r-phenyl-n-propylamino)diphenylsulphone tetrasodium sulphonate. It

^{*}The development of sulphetrone is part of a programme of work on antituberculous compounds carried out by the Therapeutic Research Corporation of Great British

is available as a cream-coloured amorphous powder containing 9-10 per cent of water. Assays in this report are in terms of anhydrous material. Promin, 4 4'-diaminodiphenylsulphone-N, N'-didextrose sodium sulphonate, is a cream-coloured product containing, when freshly prepared, 10 per cent of water, assays are also given in terms of anhydrous material.

The estimation of drugs in body fluids

The estimation of sulphetrone in body fluids has been described (Brownlee, Green, and Woodbine, 1948), and is based upon diazotization and coupling to N(1-naphthyl)-ethylenediamine hydrochloride (Bratton and Marshall, 1939) The essential points are an over-all dilution of 1 in 15 and the concentration of acid, both of which are critical Promin was estimated by the same method, which gave recoveries of some 75 per cent, to which a correction factor was applied Streptomycin in blood was estimated by the slide technique of Fleming (1943), using a susceptible haemolytic strain of Escherichia coli CN 1360 Blood, in quantities up to 05 cc, was obtained from the ear of the guinea-pig by drawing it into a clean heparinized capillary tube

EXPERIMENTAL

Eighty-three young adult guinea-pigs of mixed sexes. weighing 650-700 g, were inoculated deeply into the right thigh muscles with 0.25 mg of a fourteenday-old sub-culture of human tubercle bacillus, Seventeen days after infection the animals CN 844 were tested for their reaction to an intradermal injection of 01 cc of 1/10,000 Old Tuberculin All animals gave a positive reaction, eight reactions were classed as mild Tuberculin tests were repeated at the 15th and 23rd week of infection twenty-first day, liver samples were obtained from two animals from each group by biopsy under pentobarbital sodium anaesthesia On the following day the animals were divided into five groups, four of 18 animals and a control group of 11 animals

Streptomycin treatment

One group of 18 guinea-pigs received the equivalent of 10 mg of pure streptomycin base daily Four four-hourly intraperitoneal injections each of 25 mg of streptomycin base contained in 025 cc of pyrogenfree distilled water were given with aseptic precautions at 8 a m, 12 noon, 4 p.m, and 8 p m, no drug was given during the intermediate 12 hours ment was continued for 168 days, during which time each guinea-pig received 168 g. of the antibiotic Concentrations of streptomycin per cc of plasma were estimated at intervals throughout the test and the average figures 1 hour after injection were 70 μ g ±10 (SE of mean of 60 observations), and at 35 hours 40 μ g ± 10 (72), at 4 hours no sfreptomycin could be detected in the plasma Two animals in this group died from intraperitoneal haemorrhage as a result of accidental puncture of the spleen, one at the 7th week after infection, and one at the 8th week

Combined streptomycin and sulphetrone treatment

In addition to treatment with 10 mg of strepto mycin base daily, a group of 18 guinea-pigs also received 2 per cent of sulphetrone incorporated in the dry diet

Plasma concentrations of streptomycin were estimated at intervals throughout the test, and the average figures were similar to those of the streptomycintreated group, these were $70~\mu g \pm 1.5~(90)$ at 1 hour, and $40~\mu g \pm 1.0~(90)$ at 3.5 hours. The average blood sulphetrone concentration per 100~cc was $5.1~mg \pm 3.8~(90)$

Sulphetrone treatment

A group of 18 guinea-pigs received 2 per cent of sulphetrone in the dry diet. This corresponded to an average drug intake of 600 mg daily and resulted in an average blood sulphetrone concentration per 100 cc of 59 mg ±35 (86)

Promin treatment -

A group of 18 animals was treated with promin incorporated to a concentration of 0.5 per cent in the diet. The average drug intake was 150 mg daily and gave a blood promin concentration per 100 c c of $3.5 \text{ mg} \pm 2.2 \text{ (60)}$

Control group

Eleven animals constituted the control group and received no treatment

RESULTS

Survival times

Inoculation with 0.25 mg of the CN 844 strain established a slow chronic type of infection well suited to the comparison, for this reason only three animals in the control group of 11 died during the experiment of 168 days, and these from gross generalized tuberculosis. In the same period of time two animals died in the streptomycin treated group at 46 and 56 days owing to intraperi toneal haemorrhage as a result of needle injury to Post-mortem examination revealed the spleen An unfortunate accident minimal tuberculosis resulted in the death of five additional guinea-pigs of this group, on the 66th day, thus reducing the effective number of streptomycin guinea-pigs to eleven Post-mortem examination of these animals showed no detectable lesions Two animals in the combined streptomycin and sulphetrone group died with intraperitoneal haemorrhage owing to spleen injury, in both animals the tuberculous lesions were minimal One animal found dead on the 168th day had no tuberculosis other than a small abscess at the site of injection and an enlarged inferior inguinal gland, the cause of death was believed to have been a concurrent salmonella Four animals in the sulphetrone group infection

died, in none of them was the tuberculous process so well advanced as to justify the view that death was due to tuberculosis. Within the promin group there were seven deaths one of these, at 25 days, was due to a streptococcal pneumonia, minimal tuberculous lesions being present, in the other six animals the tuberculosis was extensive enough to justify the belief that it had been the major cause of death, a contributory feature being the toxic nature of the drug, guinea-pigs in this group were subdued and in poor condition

Effects on weight

The influence of the drug treatment on the average weights of the guinea-pigs is shown in Fig 1, where the effect of the four test substances and combination of substances is compared with the control group Both groups receiving strepto-

mycin by intraperitoneal injection show a loss of weight which lasts for some six weeks and is then followed by recovery. The fact that the final weight records show the greatest gains within this group suggests that the temporary loss of weight may have been caused by the repeated intraperitoneal injections at the commencement rather than by a specific toxic effect.

The group receiving sulphetrone gained in weight, and the group receiving combined sulphetrone and streptomycin, though they barely maintained their weight, were in good condition and more alert than any other group. The promin group lost as much weight as did the controls, with which they compared unfavourably in general condition. When the experiment ended their condition was exceedingly poor and suggestive of toxaemia.

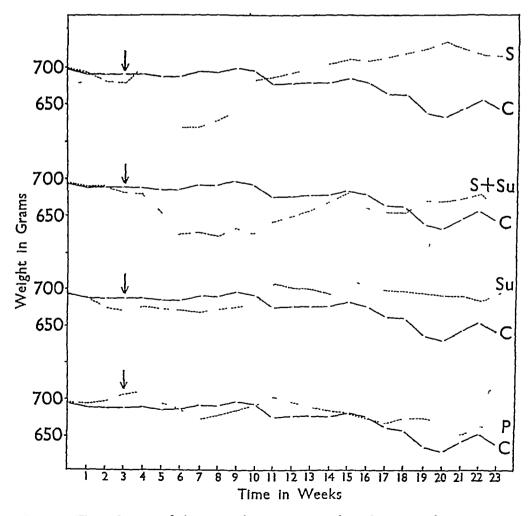


Fig 1—The influence of drugs on the average weights of groups of guinea-pigs infected with 0.25 mg of a virulent strain of human tubercle bacilli. Drug therapy begun at arrow S, streptomycin S+Su, streptomycin with sulphetrone Su, sulphetrone P, promin C, control untreated group

Clinical

Differences between the treated and untreated groups, and within the treated groups themselves, were apparent by 90 days after infection untreated group the superior and inferior inguinal glands were grossly enlarged In the groups treated with streptomycin alone and with streptomycin and sulphetrone these glands were not enlarged, or only slightly so This difference was less obvious between the sulphetrone and control groups and not evident at all in the comparison of the promin and control groups The normal appearance of the groups on streptomycin alone and on combined therapy contrasted strikingly with the toxic appearance of the group treated with promin

Tuberculin tests

Throughout the course of the experiment three tuberculin tests were made. The first, for the purpose of rejecting non-reactors, was on the 17th day, a second was made on the 105th day, and a third on the 147th day. On each occasion 0.1 c.c. of a 1 in 10,000 dilution of Old Tuberculin was injected intradermally and the subsequent reaction read at 72 hours. At the second test the severity of the reactions within the different groups appeared to be related to the extent of the disease

suggested by the records of enlarged glands, losses in weight, and general health of the animals

Reducing the clinical observations to a simple numerical basis gave the figures shown in Table I for both 105 and 147 days. The numerical notation is as follows: 1, negative, 2, query negative, or a needle injury, 3, weak positive such as mild diffuse staining of skin or a small reddened area of 3 mm or less in diameter, 4, positive, without

TABLE I
TUBERCULIN REACTIONS IN DRUG-TREATED AND
UNTREATED GUINEA-PIGS

Group	Number of	Mean numers reactions	
	guinea pigs	105 days	147 days
Streptomycin +	12	38 ± 12	45±16
Sulphetrone	16	25±09	37 ± 13
Sulphetrone Promin	15 16	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	45 ± 14 $49 + 18$
Control	11	52±16	45±14

necrosis, 5, positive, with necrosis of mild limited appearance, 6, positive, with necrosis of moderate extensive appearance, 7, positive, severe reaction. The Table shows the mean numerical indices corresponding to degrees of severity of actions

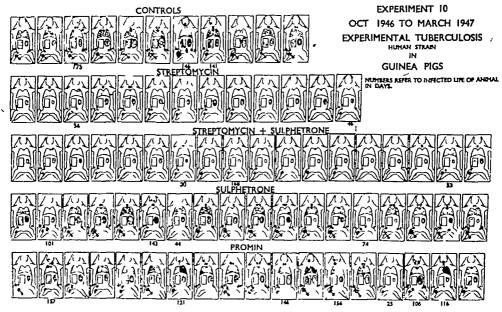


Fig 2—A pictorial representation of the degree and distribution of experimental tuberculosis in guinea-pigs post mortem 178 days after infection with 0.25 mg of a virulent strain of tubercle bacill. The drug-treated groups received 10 mg streptomycin base daily, 10 mg streptomycin base daily, 10 mg streptomycin base daily plus 2 per cent sulphetrone in the diet, 2 per cent sulphetrone in the diet, and 0.5 per cent promin in the diet. The control group was untreated

Although the disease appears to have been progressive during the course of treatment, the comparative effectiveness of each drug in limiting the spread of the disease appears to have remained constant Statistically, the differences are probably real

The enlargement in the accessory glands also shows that the disease was progressive in all groups during the interval between the second and third tests. The decrease in the tuberculin reaction in the control group is probably explained by the common observation, both in animals and man, that in the course of progressive disease skin reactions often become less sensitive

Necropsy studies

The experiment was terminated on the 178th day and the animals brought to post-mortem examination. We have become used to observing and recording differences between treated and untreated animals, but have not met previously such striking differences as were observed between the group which received combined treatment and the controls, and to a less extent between the streptomycin and control groups. In these treated groups both spleen and liver were normal in size and colour and tuberculosis was minimal in extent

There was gross, extensive, and progressive generalized tuberculosis in all animals of the untreated group. The promin treated group showed, in general, markedly less tuberculosis, although in many animals spleens were grossly tuberculous. Within this group five animals had disease comparable to that seen in the control group, with ex-

TABLE II

AVERAGE SEVERITY AND DISTRIBUTION OF TUBERCULOSIS

EXPRESSED NUMERICALLY

	Ma	croscopio		nation colved	of the t	ıssues
Group	Num ber of and mals	Spleen (maxı mum 35)	Lungs (maxı- mum 30)	Liver (mavi mum 25)	Site and lymph nodes (maxi mum 10)	Average index (maxi mum 100)
Controls	11	30 5	25 5	19	10	85 0
Strepto- mycin Strepto mycin and	13	139	46	0	9	27 5
sulphe- trone Sulphe-	18	94	2 5	0.5	83	20 7
trone Promin	18	20 0 24 2	18 6 22 2	94	9 4	57.4 69.1

tensive caseation and cavitation of the lungs The sulphetrone group showed much less tuberculosis than the control group and less tuberculosis than the promin group This was particularly evident in the spleens and liver

The extent and distribution of the disease in the drug treated and the untreated groups is shown pictorially in Fig 2 and numerically in Table II in which, following the excellent notation of Feldman (1943), scores are given for the extent and character of the tuberculosis observed macroscopically in spleen, liver, lungs, and glands

Bacteriology

Post mortem the spleens were removed aseptically and divided for histological examination and examination for tubercle bacilli For the latter purpose they were ground in a previously sterilized mortar in 6N HCl, spun, neutralized with a calculated amount of NaOH and seeded on to eggmedium Acid-fast organisms of typical morphological appearance were recovered from all the control animals, within the promin group there were 16 out of 18 recoveries, with sulphetrone 12 out of 18, with streptomycin 4 out of 13, and from the combined therapy group 2 very scanty growths from 18 cultures It was noticeable that the cultures obtained from the promin treated pigs grew luxuriantly, in contrast to all other treated groups

Sensitivity of strain and cultures to drugs

The CN 844 strain when grown in Long's Synthetic Medium is inhibited by 0.5 μ g/cc of streptomycin, by 65 μ g/cc of sulphetrone, by 125 μ g of promin, and by a mixture of 30 μ g/cc sulphetrone and 0.25 μ g/cc streptomycin, in which mixture an additive, not a synergistic, effect is seen. Significant, in view of the therapeutic effects, was the finding that all strains recovered post mortem were unchanged in sensitivity to streptomycin and sulphone drugs

Histology

Examination of histological material from the untreated control group revealed a uniform picture of severe, progressive infection. Confluent tuberculous interstitial pneumonia, with frequent caseation and many acid-fast bacilli, was seen in the lungs, the livers exhibited multiple tubercles spreading outwards from the portal tracts with many acid-fast bacilli in infarcted areas. Spleens had massive infarcts, haemorrhages, and coalescent small tubercles, mostly with caseous centres and many acid-fast bacilli were present. The

lymph nodes were occupied by confluent caseating tubercles and generally acid-fast organisms were seen

The treated groups differed, more or less, in the amount and distribution of the tuberculosis and in the numbers of acid-fast bacilly seen

The promin group differed least from the controls Involvement of the lungs was the rule, but there was evidence of localization and repair Livers showed little frank tuberculosis, but there were extensive foci of recent necrosis, acid-fast organisms were rarely seen. Spleens were extensively involved, with numerous tubercles, extensive infarction and congestion acid-fast organisms were tare.

The restraining powers of the sulphone drugs were carried a demonstrable step forward in the sulphetrone group. Lungs were less extensively involved, the lesions being limited at the most to patchy tuberculous interstitial pneumonia coalescing in areas into large tubercles, with rare necrosis, at the least, there was congestion and oedema, acid-fast bacilli were not seen. Livers showed small areas of miliary tubercles and an occasional fibrosing tubercle, acid-fast bacilli were not seen. Spleens showed numerous small tubercles in a congested oedematous pulp but without acid-fast bacilli being identified.

The streptomycin group differed markedly from the controls Only 5 out of 13 lungs showed tuberculous lesions in two they were restricted to single fibrotic calcified tubercles. In only one animal was there a tuberculous lesion in the liver. With the exception of one animal in which the disease appeared to be non-progressive, tubercles were found in all spleens. Mostly the tubercles were fibrotic (5 cases), or calcified (7). Tubercles were seen in all the contiguous lymph nodes, in three animals they were limited to small fibrotic areas.

The group treated with streptomycin and sulphetrone differed remarkably from the controls and presented an impressive degree of protection even when compared with the streptomycin group Only 6 out of 18 lungs showed tuberculous lesions in five of these the lesion was calcified and fibrotic All lungs showed evidence of many healed lesions In only one liver was a tubercle found and this was a small hardened epithelioid node The spleens were involved in 14 out of 18 animals, but all the lesions were non-progressive, consisting of areas of diffuse fibrosis or of repressive lesions with calcification Lymph nodes adjacent to the inoculation site were minimally involved in all animals, all the lesions were non-progressive and

consisted almost entirely of fibrous tissue with some calcification

DISCUSSION

Although incapable of eliminating the causative organism unconditionally, streptomycin is capable of modifying favourably the course of inoculation tuberculosis in the experimental animal (Smith and McClosky, 1945, Feldman, Hinshaw, and Mann, 1945, Youmans and McCarter, 1945) Viewed from the base-line laid down by experimental and clinical sulphone therapy the significant advance in efficiency made by this new antibiotic justified a careful clinical assessment. From the clinical reports now available it is apparent that the antibiotic is able to influence tuberculosis to only a limited extent and under certain conditions Ironically enough these appear to be the conditions under which the bacillus is multiplying rapidly, for example in miliary tuberculosis The importance of appreciating the reasons for these failures needs no stressing Thus, Baggenstoss, Feldman, and Hinshaw (1947) record the death of 5 cases of miliary tuberculosis in spite of healing in the lungs, and they note that streptomycin could not be demonstrated in brain substance in which tuberculous lesions were found Madigan, Swift, and Brownlee (1947b) had similar failures with miliary tuberculosis to report, and in addition they could not record healing in phthisis of diverse origin If the disease in the experimental animal is regarded as different from the diseases due to M tuberculosis in man, this difference reflects the metabolic adaptability of the organism and it is to countering this aptitude that we must address our The acute experiments, here described, offer no evidence of adaptation of the organism to any of the chemotherapeutic agents, significant in this connection is the record of Madigan et al (1947b), that in chronic disease (phthisis) the organisms acquired resistance to streptomycin, while in acute infection (miliary tuberculosis) in which healing was seen the susceptibility of the organism Experimental evidence is prewas unchanged sented by the same authors, that streptomycin is most effective against young dividing bacilli and ineffective against old resting cells A similar observation is made by Middlebrook and Yegian A practical approach to the attack on the (1946)metabolic adaptability of the tubercle bacillus is that of Smith and McClosky (1945), who showed the synergistic action of streptomycin and promin in experimental tuberculosis, Callomon, Kolmer, Rule, and Paul (1946) also demonstrated a similar effect with diasone and streptomycin

The present report is concerned with the synergism of streptomicin and sulphetrone, a diamino-diphenylsulphone derivative which combines efficiency of action with freedom from the chronic toxicity associated with the use of promin and diasone. Groups of animals treated with promin, with sulphetrone and with streptomicin enable a comparison to be made

On the basis of survival time, change in weight, response to tuberculin tests, macroscopic evidence of gross tuberculosis post mortem or microscopic examination there is presented a uniform picture of the marked superiority of the combined therapy The order of efficiency is streptomycin with sulphetrone streptomycin, sulphetrone, and It should be noted that oral promin exhibits the drug by its most toxic route and that the resulting blood levels are consequently low, this remains, however a practical prob-It is interesting that a relation was found lem between the degree of protection and the severity of intradermal tuberculin reactions, an observation in contrast to that of Smith and McClosky (1945) A careful sifting of all evidence here presented leaves the impression that the disease was progressive in all groups, albeit at a much suppressed rate in those groups where protection was greatest Nevertheless with this implication in mind the experimental effects produced by the combined streptomycin and sulphetrone therapy are believed to justify a careful clinical evaluation in selected The need meanwhile is for improved antituberculous bacterial antibiotics which will not induce resistant strains

SUMMARY

1 Four groups of 18 guinea-pigs, and one control group of 11, infected with 0.25 mg of a human virulent strain CN 844 of *M tuberculosis* were treated 22 days later with drugs for 168 days. One group received 0.5 per cent of promin in the diet, one 2 per cent sulphetrone in the diet, one 10 mg of streptomycin parenterally daily, and a fourth 2 per cent sulphetrone and 10 mg of streptomycin. The control group of 11 animals remained untreated

2 Twice during treatment all animals were tested for reactions to the intradermal injection of Old Tuberculin There appeared to be a simple relation between the degree of severity of the intradermal reactions and the course of the disease

judged by weight records and enlargement of lymph nodes

- 3 Records of losses in weight, enlargement of glands, relation to tuberculin tests, macroscopic evidence of distribution of tuberculosis post mortem, and the evidence of histology, all presented a uniform picture of degrees of protection. All drug-treated groups showed evidence of protection. It was least in the promin group, became greater in the sulphetrone group, still greater in the streptomycin group, and was greatest in the group treated with sulphetrone and streptomycin. In the latter group the protection was so marked as to be clearly synergistic.
- 4 The opinion is expressed that the disease was progressive in all groups
- 5 Strains of the tubercle bacillus recovered from the drug-treated groups were unchanged in their susceptibility to the antibacterial action of the drugs
- 6 The experimental effects produced by combined streptomycin and sulphetrone therapy are believed to justify a careful clinical evaluation in selected cases

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THE CHEMOTHERAPY OF AMOEBIASIS

PART I INTRODUCTION AND METHODS OF BIOLOGICAL ASSAY

BY

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Three types of drug are commonly used for the treatment of amoebiasis (a) emetine or its sparingly soluble derivatives such as the bismuth iodide ("EBI"), (b) halogenated quinolines such 7 iodo-8-hydroxyquinoline-5-sulphonic (chiniofon BP "yatren," "quinoxyl"), 5-chloro-7-iodo-8-hydroxyquinoline ("vioform") and 5 7disodoquinoline ("diodoquin"), and (c) arsenical preparations such as 4-carbamidophenylarsonic acid (carbarsone) and 3-acetamido-4-hydroxyphenylarsonic acid (acetarsone BPC, "stovarsol") It is usual in current practice to administer courses of these drugs together with retention enemata of chiniofon Recently penicillin and sulphonamides have also been given to control secondary infections and this treatment has been successful in a number of cases previously resistant to treatment with amoebicides alone (Hargreaves, 1945a and b, 1946)

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In spite of this armoury of medicaments there is still a great need for a new drug, not only to deal with refractory cases, but also if possible to avoid the objectionable properties of emetine Ipecacuanha is scarce and expensive, and its most important alkaloid, emetine, although highly specific, is toxic and even in its pharmaceutically most elegant forms causes nausea and vomiting in many patients. The systematic search for new amoebicides has been hampered by the lack of a reliable in vitro test and of a convenient experimental infection in animals.

Pyman (1937a and b) examined a homologous series of harmol ethers (I) in which R was an alkyl group containing from two to twelve C atoms

A peak in amoebicidal activity

in vitro was found with R=C,H,, but the compounds were very sparingly soluble In order to

increase the solubility, basic groups were introduced into the terminal position of the alkyl groups $[(I),R = Alk_2N(CH_2)_x]$, the number of methylene groups and the size of the alkyl groups were varied and a peak in activity was found in O-11-din-butylamino-undecylharmol $[(I), R = (C, H_0) N$ (CH₂)₁₁-] which, under the conditions of the test used, was lethal to Entamoeba histolytica in a dilution of 1 750,000 to 1 4,000 000 As this was many times more active than O-n-nonylharmol it was conjectured that the harmol residue might not play an important part in the amoebicidal action, and this part of the molecule was replaced by other groups, leading ultimately to the preparation of 1 10-bis-(di-n-amylamino)-decane (II) which was found to be three to five times as efficient as emetine Unfortunately a clinical trial failed to confirm the promise of the in vitro test. It is evident from this careful work of Pyman and his collaborators that an in vitro test alone is not enough to indicate whether or not a compound is likely to be useful in the treatment of amoebiasis

The present series of papers describes an attempt to synthesize amoebicidal substances based upon the structure of emetine, and upon the structure of certain compounds found by Pyman to be active in vitro. Details of the methods used for biological tests, and the results obtained with standard amoebicides will be described in the present paper, and an account of the new compounds prepared and their biological activities will be given in Parts II and III

IN VITRO AMOEBICIDAL TESTS

The activity of ipecacuanha alkaloids upon freeliving amoebae was demonstrated by Vedder (1911) and by Pyman and Wenyon (1917), but satisfactory evidence of specific activity against *E histolytica* was not forthcoming until a medium was devised which would maintain the amoebae alive sufficiently long for the drugs to take effect, and which contained no solid phase to remove the alkaloid from solution. The buffered horse-serum Ringer medium (hs) of Laidlaw, Dobell, and Bishop (1928) met these requirements and was used by Pymin and his collaborators (1937a and b) for the examination of new compounds. A further source of difficulty has been the fact that so far it has not been possible to grow E histolytica in the absence of bacteria, and the results of in vitro tests have been complicated by the presence of un indeterminate mixed bacterial flora used by the amoeba as a source of essential metabolites American work (Rees Reardon Jacobs, and Jones 1941) has shown that it is possible to grow the organism in the presence of single strains of bacteria In this country Dobell has succeeded in growing E histolytica in vitro in the presence of a single pure strun of Bact coli, and it was reported by Hargreaves (1945) that in such a culture the amoebae were killed by a concentration of emetine hydrochloride as low is 5,000,000 The use of this culture enables the observer to detect bactericidal action of a new compound which might be confused with direct amoebicidal action in a culture containing many strains of bacteria, some but not all of which were affected by the drug. The work of Pyman and the more recent work of Rawson and Hitchcock (1947) suffer from the disadvantages resulting from the presence of a mixed bacterial flora

Methods used in the present work

For the cultures used, and for the method of test described below, we are indebted to Dr Clifford Two strains of amoeba have been used, Dobell both growing in culture with a single strain of Bact coli One of them was obtained from a natural infection in a monkey, and was morphologically indistinguishable from E histolytica, the These strains were other was isolated from man identical in their reaction to emetine in vitro cultures were maintained on the horse-serum Ringeregg (HSre) medium of Dobell and Laidlaw (1926) For an in vitro test, a series of 4 in $\times 1/2$ in tubes were prepared, each containing 45 ml of buffered horse-serum-Ringer medium (hs) and 05 ml of a sterile solution of drug in buffered Ringer Each was inoculated with 6 drops (0.2 ml) of a heavy culture of Bact coli in Douglas-Hartley broth, and a large loopful (20 mg) of dry, sterile rice starch added The tubes were incubated overnight at 37°C for growth of the bacteria to take place, and for the conditions at the base of each tube to become anaerobic may be confirmed in a sample tube by the addition of methylene blue before incubation, if growth is satisfactory the dye is bleached at the bottom of the tube A heavy suspension of E histolytica was then added to each tube, the moculum being placed carefully on to the surface of the starch

with a pipette. Luge quantities of inoculum were obtained by culture in flasks of HSre medium prepared by the method of Frye and Meleney (1935). After incubation for three days, the tubes were examined microscopically for the growth of amoebae, the bacteria present tested for viability by subculture and the pH of each tube of culture checked. The results obtained with standard amoebicides are recorded in Table I.

TABLE I

THE ACTIVITY OF AMOEBICIDAL DRUGS in vitro

Drug	Gro of	Growth of amorbae in the presence of various concentrations of drug											
Drug	10	-4 10-4	10-4	10-7									
1 metine HCl Carbarsone			± +	++									
Acethrone Climiofon Diodoquin*	1 -	· +	' + +	+ + +									
-		1		1									

*Almost insoluble, solid particles present

Note of these drugs had bactericidal activity at the dilutions used

IN VIVO AMOEBICIDAL TESTS

Still greater difficulties have been encountered in the finding of a suitable experimental infection upon which the *in vivo* activity of drugs could be investigated. Thousands of cats have been used in some laboratories (Wagner, 1935, Bieling, 1935, Kikuth, 1945) in spite of the fact that Dale and Dobell had shown in 1917 that the acute and lethal infection produced in kittens by the inoculation of *E histolytica* per rectum was not affected by doses of emetine large enough to be toxic to the host, and that therefore experiments with new drugs could not be expected to yield much useful information

Several workers have reported infection of rats and mice by the oral inoculation of E histolytica cysts but such experiments have not been uniformly successful in the past Meleney and Frye (1932) and Deschiens and Provost (1937) described the infection of cats by the direct inoculation of trophozoites into the lower ileum in a recent paper by Jones (1946) this method has been modified and applied successfully to young rats, and a method of testing drugs has been carefully worked Our own experiments made independently during the past two years and described in the present series of papers produced similar results, showing that the young rat is a suitable host for caecal infections of E histolytica The infection in the rat caecum differs from that in the human

gut in that we have never yet seen an ulcer of the deep flask-shaped type in which amoebae are living exclusively upon the tissues of the host. The ulcers are extensive but shallow and rarely involve tissues deeper than the mucosa. The active amoebae frequently contain bacteria, whereas ingestion of red cells is only occasionally encountered, and cysts are rare. Nevertheless, as infected rats can be cured by the drugs used in human therapeutics, we consider the method to be the best so far devised for the investigation of new compounds designed as amoebicides.

Methods used in the present work

Animals

Very young rats weighing 20-30 g were used immediately after weaning. At this age the animals have not developed an extensive caecal flora and fauna, having had milk as their main diet. The caecum is small and almost empty apart from a mucous secretion. At a later age the caecum becomes enlarged and distended with food residues and living organisms, and the inoculation of E histolytica is less likely to produce an acute infection. Chronic light infections could be produced in older rats, but were less suitable for therapeutic experiments

We were troubled in the early stages of this work by natural infections of the rats by an amoeba resembling E muris This organism is distinguishable from E histolytica in stained smears, but is difficult to detect in fresh preparations especially when E histolytica is also likely to be present. We also had the impression that a rat infected with E muris was difficult to infect with E histolytica, caecal conditions favourable to the former being apparently unsuitable for the latter Experiments in these laboratones by Neal (1947) showed that hamsters freharboured heavy caecal infections quently E muris and discharged cysts which were infective They therefore served as a reservoir from which young rats readily became infected Rats could be kept free from infection by keeping them in a room apart from hamsters and by bedding them in clean wood-wool sterilized by heating at 150°C for 1 hour

Strain of amoeba

We have tried a number of strains isolated from human patients, and find them to be variable in their pathogenicity to rats. It is possible that this is caused by differences in associated bacterial flora. The strain we have used for routine tests ("Strain B") was derived from a man infected in Burma, and had resisted a total of 11 courses of treatment with emetine and other drugs before it reached our hands. We are indebted to Air Commodore T. C. Morton for the material. This organism when grown in bulk upon HSre medium and inoculated intracaecally caused infection of 80 to 100 per cent of young rats. We did

not find it necessary to add mucin to the inoculum as recommended by Jones (1946)

Method of inoculation

The rats were lightly anaesthetized with ether, the skin of the abdomen shaved, and the caecum exposed through a very small incision slightly to the left of the mid-line A volume of 0.2 to 04 ml of a nch suspension of amoebae from culture was injected directly into the caecum, the jet of fluid being directed in such a way that the blind end of the caecum was completely filled and good contact was made with the mucosa A small amount of a mixture of equal parts of sulphanilamide and marfanil (about 10 mg) was dusted into the abdominal wound to minimize chances of secondary infection, and the wound was closed with a rayon suture in the muscle and a Michel clip in the skin. The dusting with sulphonamides has no effect upon the development of the amoebic infection

Treatment with drug

The rats were divided into groups and fed for six days upon diet containing the drug to be tested diet was a Coward stock diet, and the amount of drug incorporated was determined by the results of toxicity The daily food consumption of a newly weaned rat is about 4 g per day and the maximum concentration used for any drug was 05 per cent, representing a daily dose of about 800 mg per kg advantage of the drug-diet technique is that the drug may be distasteful to the rats, and the food intake is then less than normal This can readily be detected by observation of the food consumed and if a new drug shows promise as an amoebicide, more accurate treatment can be ensured by frequent dosage by The drug-diet method is convenient for catheter "screening" tests and ensures continuous treatment with drugs A group of untreated infected controls was used in every test to check the infectivity of the ınoculum

Assessment of results

For the assessment of the effect of a drug, the criterion we used was the presence or absence of amoebae in the caecum, as demonstrated by micro scopical examination and by culture in HSre medium Caecal contents showing plentiful amoebae under the microscope will not always grow out in culture if the concomitant bacterial flora is unsuitable, but culture will sometimes demonstrate the presence of an infec tion not detected microscopically, and is thus a useful supplement to direct examination The condition of the caecum of each rat was also assessed by a "scoring" method similar to that described by The presence of amoebae did not Jones (1946) enter into our scores, which were awarded upon The conditions of the macroscopical evidence only caecal wall (normal=0, extensive ulceration=4) was scored separately from the condition of the contents

(normal 0 mucus only = 4) We have found that a drug may provide marked protection of the enecal wall and yet not remove the amouble from the contents another drug may remove the amouble, but as a result of its irritant action the case it wall will appear thickened and the contents fluid or mucoid Such examples cannot be scored by Jones's method and we consider that the alternative system described above gives a better analysis of the effects of a drug

The results obtained with a series of standard amoebicides are shown in Table II

TABLE II
THE ACTIVITY OF AMOUNTAIN DRUGS IN 1410

	Con		tion of rum		t from
Drug	centri non in diet %	Will (mean score)	Con tents (mean score)	Pro por tion	Per cent
Normal rits Infected controls	_	30	0 2 8	30/156	19
Fractine HCl	0 002	02	10	16/19	89
	0 001	06	05	12/15	90
	0 0005	13	09	2/9	25
	0 00025	13	13	0/4	0
Emetine bismuth rodide	0 05	19	17	9/9	100
	0 02	15	28	26/29	90
	0 01	10	17	27/30	90
	0 005	17	19	15/17	88
	0 002	20	21	1/7	14
Carbarsone	0 4	0	0 3	6/6	100
	0 2	1 6	1 7	10/22	45
	0 1	2 1	2 1	3/8	38
Acetarsone	0 4	0	0 6	7/7	100
	0 2	1 0	1 2	12/23	52
	0 1	1 3	1 1	3/8	38
Chiniofon	0 2	0	0	8/8	100
	0 1	2 8	2 5	4/17	24
	0 05	3 6	2 6	0/7	0
Diodoquin	0 5	0	0	8/8	100
	0 2	3 0	2 9	4/12	33
	0 1	2 0	3 0	2/9	22

DISCUSSION

The activities of drugs *in vitro* shown in Table I demonstrate the highly specific action of emetine. The limiting amoebicidal concentration of 10 to 10 to 10 to 10 is in agreement with the findings of Dobell (reported by Hargreaves, 1945, 1946). All the other drugs were much less active, or showed no activity at 10-to The results of the *in vivo* tests (Table II) also show emetine to be the most active drug tested in spite of the fact that the strain was derived from a case which had resisted repeated courses of this and other drugs. Emetine bismuth 10dide showed activity commensurate with 1ts alka-

loidal content the inorganic fraction appeared to have no influence upon the potency of the drug in The results of the acute experimental infection giving emetine in the diet were more successful than those reported by Jones (1946) in which subcutaneous injection of six doses of 2 mg/kg, or oral administration of a single dose of 12 or 15 mg/kg protected only 60 per cent or less of the animals * A drug diet containing 0 001 per cent of emetine HCl, corresponding to a daily intake of approximately 1.5 mg/kg was sufficient to protect 12/15 rats infected with strain "B," though we have evidence that strains derived from other sources may be less susceptible to emetine Rats receiving drug diet containing 0.02 or 0.05 per cent of EBI showed considerable irritation of the cacca, the walls appearing thickened and pale and the contents fluid. This is shown by the higher scores at these dose levels

Comparison of Tables I and II shows that the drugs tested fall into the same order of relative activity by both in vitro and in vivo methods. Experiments with other drugs, which will be recorded in Parts II and III, have shown that this is not always the case. In the past it was a common practice to calculate the "chemotherapeutic index" of a drug from the ratio

"maximum tolerated dose"
'minimum curative dose"

These terms have fallen into disfavour because their errors are large and indefinable and an attempt to provide a more accurate index has been made by expressing it as the ratio of the median lethal and median effective doses (Wien, 1946) Dr J W Trevan, at the January, 1947, meeting of the British Pharmacological Society, pointed out that such a ratio was of little value if the doseresponse curves were not parallel, and also that itdid not express the relationship required—namely, a comparison of the safe dose with the curative dose He suggested that a better figure would be given by the ratio of the "LD 01" and the "CD 999," which can be regarded as accurate substitutes for "maximum tolerated" and "minimum curative" doses These figures may be determined from the dose-response curves by extrapolation or by calculation from the regression formulae

In Table III this method has been applied to the amoebicides Toxicity determinations were made upon rats of the same age and strain as those

^{*} Added in proof In a more recent paper Jones (1947) reports results from repeated oral administration of drugs which are very similar to those found by us

used for amoebicidal tests, and the figures were obtained graphically The total daily dose commonly employed in human therapeutics is also recorded, and will be seen to compare reasonably well with the daily intake required to cure rats

TABLE III

Drug	Maxi- mum clinical daily dose mg/kg	Acute toxicity to young rats LD 0 1 mg /kg orally	Amoebici dal activity CD 99 9 mg /kg /day in diet	Chemo thera peutic index LD 0 1 CD 99 9
Emetine HCl Emetine bismuth iodide Curbarsone Acetarsone Chimofon Diodoquin	3 14 10 30 30	6 11 4,500 1,200 800 >40 000	4 5 15 600 600 300 600	1 3 0 73 7 5 2 2 7 >67

Table II shows that, in spite of their specific activity, it was difficult to obtain 100 per cent of cures with emetine HCl or emetine bismuth iodide This was probably because emetine drug-diets containing more than about 0 001 per cent are irritant and distasteful to rats, the total dose therefore may not have been taken each day. On the other hand individual rats may carry infections which resist treatment with the alkaloid In either event the question requires further investigation CD 99 9 values for these two drugs were estimated by extrapolation of the linear parts of the doseresponse curves and are therefore probably too low Reference to Table III shows that even these low figures are for emetine bismuth iodide within. and for emetine hydrochloride almost within, the toxic range Chiniofon is the next most active, and also the next most toxic drug Carbarsone appears to be better than acetarsone because of its lower toxicity, and diodoquin has the highest chemotherapeutic index of all because no rats died after an oral dose as high as 40 g/kg has no appreciable activity in vitro even when solid particles of drug are present (Table I), it is probable that diodoquin is converted to a more active substance during its passage through the body

In clinical practice none of the drugs is successful in every case

SUMMARY

- 1 Methods are described for the comparison of the amoebicidal activities of drugs (a) in vitro, using a strain of Entamoeba histolytica growing in the presence of a single strain of Bacterium coli, and (b) in vivo, using young rats inoculated intracaecally with cultures of amoebae
- 2 The following standard amoebicides have been tested emetine, emetine bismuth iodide, carbarsone, acetarsone, chiniofon, and diodoqu n Emetine was the most active, both *in vitro* and *in vitro*
- 3 The chemotherapeutic indices of the drugs have been calculated and show that the curative dose of emetine is very close to the toxic dose. Of the drugs tested, diodoquin had the most favourable chemotherapeutic index

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THE CHEMOTHERAPY OF AMOEBIASIS

PART II AMINES DERIVED FORMALLY FROM EMETINE

BY

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The structure of emetine according to Brindley and Pyman (1927) is probably represented by (I) If the bond shown by the dotted line at (a) in (I) is considered to be broken, a structure is derived consisting of two 6 7-dimethoxytetrahydroiso-quinoline nuclei joined at the 1 1'-positions by a chain of five methylene groups bearing two branches. Child and Pyman (1929) have synthesized a series of $a\omega$ -bis(6 7-dimethoxytetrahydroisoquinolyl) alkanes of formula (II) based on this model and found the compounds to be inactive against Entamoeba histolytica in vitro

We have prepared a series of compounds (III, n=6 to 10) modelled on the bis (β -3 4-dimethoxyphenylethylamino) alkanes resulting from the further rupture at (b) (b) of the ring system (II) The nature of the substituents in the benzene ring has been varied, and also the length of the central chain of methylene groups and of the chain between the amino group and the benzene ring

A series of bis(alkylamino)alkanes has also been prepared, the formal relationship of which to emetine is exemplified in bis(octylamino)heptane (IV) This structure is derived from that of emetine by opening out the molecule at the bonds (a), (b) (b) and (c) (c) in formula (I) In this series the length of the central chain of methylene groups has been varied and the chain branched, and the terminal alkyl groups have been varied in a similar manner

All these substances have been tested for amoebicidal activity by the methods outlined in the first paper of this series (Goodwin, Hoare and Sharp, 1948), and the results are shown in Table I Selected members of the series have also been tested against experimental infections with trypanosomes, leishmania and malaria. The chemical syntheses and characteristics of the compounds are described in the chemical section on page 56

As the quantities of drug available have in some cases been limited, the numbers of animals used were small. The results of the toxicity tests are very approximate, having been calculated by Karber's method (Irwin and Cheeseman, 1939) from three or four groups of 5 mice. It will be

TABLE I

Columns 6 and 7 "+" signifies improvement, "-" no	Column 12	"1" st	gnific	s no a	ctivity a	againsi	T equiperdum
improvement.		"2"	,,	**	• • • • • • • • • • • • • • • • • • • •	**	T rhodesiense
Columns 10 and 11 "-" signifies no activity at a concen-		"3"	,,	,,	,,	"	T congolense
tration of 10 ⁻⁴		"4"	,,	,,	,,	,,	T cruzi
Figures in parentheses denote slight activity with large doses		"5"	,,	,,	,,	"	Leishmania
(e g "(6)" for compound No 13 means that there was							donos am
slight antimalarial activity at toxic dose levels)		"6"	,,	,,	,,	,,	Plasmoduum
							gallınaceum

	Approx LD50			Amo	pebicio	ial te	sts		1		
	Ref	(mg.	/kg)			ın vivo	,		in v	itro	- Other
Substance	No	Oral	Sub- cut	diet		aecal dition	Rats		Amoe- bicidal conc	Bact -cıdal conc	tests
(1) -	(2)	(3)	(4)	(5)	Wall (6)	Conts (7)	(8)	(9)	(10)	(11)	(12)
$\left(\begin{array}{c} \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \end{array}\right) \left(\text{CH}_2\right)_2\text{NH-} \left(\begin{array}{c} \text{CH}_2\right)_6, \text{ 2HBr} \\ \text{2} \end{array}$	1	950	250	`0.2 0 1	+	+	4/7 2/8	60 25	_	_	1,3, 5,6
,, (CH ₂) ₆ , 2C ₃ H ₆ O ₃	2			0 5 0 05	_	_	5/6 1/6	80 15			
,, (CH₂)₁, 2HBr	3	250	150	0 2 0 1	=	=	4/7 1/6	60 15			1,6
,, (CH ₂) ₈ , 2HBr	4	600	200	0.2 0 1 0 05	-	 	4/7 3/7 1/7	60 40 15		_	1,6
,, (CH₂)₀, 2HBr	5	600	200	0 2 0 1 0 05			1/8 4/8 1/8	10 50 10	10-4		6
,, (CH ₂) ₁₀ , 2НВг	6	700	550	0.2 0 1 0 05	+ + -	+	3/4 2/7 1/7	75 30 15	10-5	_	3,6
(CH ₂) ₂ NH-) ₂ (CH ₂) ₆ , 2HBr	7			0 2		_	0/4	0	10-4		
,, (CH ₂) ₁₀ , 2НВг	8	30	40	01	_		0/4	0	-		1,3
(HO_(CH_).NH-) (CH_), 2HCI	9		310	02		-	0/4	0	_	_	1,3
,, (CH) ₁₀ , 2HCl	10	>2000	375	02	-		0/6	0			
(CH ₂) ₂ NH-) ₂ (CH ₂) ₆	11	>2000		0 5	+	+	4/6	65	_	-	6
,, (CH₂)10	12	>2000	>2000	05			0/6	0			2,3
(Cl(CH_2_NH-)_(CH_3_, 2HBr	13	>2000	>2000	0 2 0 1 0 05	+		4/6 0/5 0/7	65 0 0	10-4	10-4	(6)
, (СН <u>.</u>) ₈ , 2НВг	14	>2000	>2000	0.2 0 1 0 05	+	-	9/9 8/15 4/14	100 55 30	10-8	_	- -

TABLE I-continued

		App	170\ 050	Amoebicidal tests							
-	Ref	(mg				111 1110			ın yı	tro	Other
Substance	No	Oral	Sub- cut	% diet	cond	ecal lition	Rats clear		Amoe- bicidal conc	Bact. -cıdal conc	tests
(1)	(2)	(3)	(4)	(5)	Wall (6)	Conts (7)	(8)	(9)	(10)	(11)	(12)
(CH-) NH-)(CH ₂) ₁₀ 2C ₂ H ₆ O ₃	15	450	> 2000	05 02 01	То	xic -	To 2/5	xic 40	10-5	_	6
, Bismuth iodide (25 3% base)	16			0 5 0 2	++	++++	5/6 3/7	80 40			
(CH ₂)_NH-)(CH ₂) ₆ , 2C ₃ H ₆ O ₃	17	742	375	05 02 01	To + +	ліс Н Н-	6/6	100 50	10-ª	10-4	
,, Bismuth iodide (26% base)	18			0 5 0 2	+	++	4/6 2/8	65 25			
$(CH_2).NH_1$ $(CH_2)_{10}$ $2C_2H_4O_7$	19	536	134	0 5 0 2 0 1	Toxic	+++	3/4 1/7	75 15	10-5	10-4	6
,, Bismuth iódide A (36 6% base)	20			0 5 0 2	+	+	5/5 1/8	100 10			
,, Bismuth iodide B (22 9% base)	21			0 5 0 2	+ -	+	5/8 0/8	60 0			
(CH ₂) ₂ NH- (CH ₂) ₆ , 2HBr	22	120	350	0 5 0 2		_	Toxic 2/7	30	10-5		(1),3, 5
,, ,, 2C₂H₀O₃	23			0 2 0 1 0 05		- -	0/3 1/6 2/6	0 20 30			1,3
,, (CH₂) ₇ , 2HBr	24	165	400	0 5 0 2 0 1 0 05	Toxic + - -	+	4/8 0/8 1/6	50 0 15	10-6	_	(1)
,, (CH₂)8, 2HBr	25	200	550	05 02 01	+ + -	+++	Toxic 9/12 2/7	75 30	10-5	-	
,, (CH₂)₀, 2HBr	26	135	68	0 5 0 2 0 1 0 05	+ +	+ +	Toxic 7/8 7/8 2/8	90 90 25	10-0	-	
,, (CH ₂) ₁₀ , 2НВг	27	450	700	0 2 0 1 0 05	++	+ + -	3/5 2/4 3/5	60 50 60	10-6	_	1
$(CH_2)_3NH (CH_2)_8$, $2C_3H_6O_3$	28	710	410	05	+	+	Toxic 3/7	40	10-5		

TABLE 1-continued

		App	rox			Amoe	bıcıdal	tests			T
	D.f	LD (mg/			i	n vivo			in vitr	О	
Substance	Ref No	Oral	Sub- cut	% diet	Ca	ecal lition	Rats	%	Amoe- bicidal conc	Bact -cidal conc	Other tests
(1)	(2)	(3)	(4)	(5)	Wall (6)	Conts (7)	(8)	(9)	(10)	(11)	(12)
$(CH_2)_5NH - $ $(CH_2)_{10}, 2C_5H_6O_5$	29	1400	700	0 5 0 2	To +	xic +	0/4	0	10 5		
$(CH_2)_1NH - (CH_2)_8, 2C_3H_6O_3$	30	308	310	02	_	_	1/6	15	10-5	_	
,, (CH ₂) ₁₀ , 2C ₃ H ₆ O ₃	31	>2000	>2000	0 5 0 2	++	++	Toxic 4/8	50	10-5	-	
$(CH_2)_6NH (CH_2)_8$, $2C_3H_6O_3$	32	_		02	-	_	3/7	40	10-5	_	
(CH ₂) ₈ , 2HCl	33	1400	350	0 5 0 2	-	_	3/7 5/7	40 70	·		
CH ₂ -O (O-CH ₂ NH-)CH ₂) ₈ 2HCl	34	1400	1400	0.2 0 1	++	+ +	8/13 3/7	60 45	_	_	
CH ₃ O CH ₂ NH- (CH ₂) ₈ , 2HCl	35			0 5 0.2	-	 - -	2/8 0/7	25 0	10-4	_	
$\left(\text{CH}_{5}\text{O}\right)$ $\left(\text{CH}_{2}\text{NH}-\right)_{2}$ $\left(\text{CH}_{2}\right)_{6}$, 2HCl	36			0 5 0 2		_	2/7 3/6	30 50	10 ⁸		
(CH ₃) ₃ N CH ₂ NH-) ₂ (CH ₃) ₆ , 4HCl	37	350	175	02	_	-	3/8	40	10-4		
(C ₇ H ₁₆ NH-)_(CH ₂) ₆ , 2HBr	38	450	100	0 2 0 1	+ +	++	4/7 4/10	60 40	10-	10 4	(1),3, 5,6
,, 2C₃H₀O₃	39		154	0 5 0 2 0 1	+++	+++	5/5 1/5 2/7	100 20 30	10-5	10-4	(1),4, 5,6
(C ₇ H ₁₈ NH-)_(CH ₂) , 2HBr	40	350	300	0 2 0 1 0 05	+	+ -	3/6 5/15 4/9	50 33 40	10-8	10 4	(1)
,, (CH) ₈ , 2HBr	41	450	350	0.2 0 1 0 05	++++	++++	4/8 3/5 1/4	50 60 25	10-5	10-1	1,6
,, ,, 2C₂H ₆ O₃	42	\		02	_	_	1/2				
,, (CH₂)₅, 2HBr	43	450	450	0 2 0 1 0 05 0 025 0 0125	+	xic + - -	12/16 3/12 5/11 0/5	25 45	10 4	-	6

TABLE 1—continued

		/DLL 1	conti	nuca							
		Apr	rox 050		Amoebicidal tests						
	Ref	(mg	/kg)			ın vivo			111 3	itro	- Other
Substance	No	Oral	Sub- cut	% dict	cor	accal idition	Rats		Amoe- bicidal conc	Bact -cidal conc	tests
(1)	(2)	(3)	(4)	(5)	(6)	Conts (7)	(8)	(9)	(10)	(11)	(12)
(C-H ₁₈ NH-)_(CH ₂) ₁₀ 2HBr	44	500	350	0 2 0 1 0 05 0 025 0 01	- - -	1 + 1 - 1 - 1 - 1	8/9 3/9 4/4 1/8 2/8	90 35 100 10 25	10 5	10 4	1,3, 5,6
(C ₆ H ₁₃ NH-) ₂ (CH ₄) ₁₀ 2C ₃ H ₄ O ₅	45	1000	270	05	+		Toxic Toxic 3/8		10 4	-	
(C _E H ₁ -NH-) ₂ (CH ₂) _E 2HBr	46	1800	>2000	0 2 0 1 0 05 0 01	+	+	11/14 1/6 7/23 6/14	15 35	10 5	10-4	1,3
, ,, 2C₃H₅O₃	47	615	>2000	0 5 0 2 0 1	+	++-	Toxic 4/4 2/6	100 35			
(C ₂ H ₁₀ NH-)_(CH ₂) ₆ 2C ₂ H ₆ O ₃	48	700	> 2000	0 2 0 1	+	+	Toxic 1/5	20	10 4	_	
(C ₁₁ H ₂₂ NH)_(CH ₂) ₁₀ , 2HBr	49	>2000	>2000	02	_		Тохіс				
(C ₄ H ₉ CH(C ₂ H ₆)CH ₂ NH-)_(CH ₂) ₁₀ , 2HBr	50	>2000	>2000	02	<u> </u>	-	0/3	0	10-5	-	6
((CH ₂ = CH-CH ₂) ₃ C NH-) ₂ (CH ₂) ₁₀ , 2HCl	51	1400	2000	0 5 0 2	++	++	Толіс 3/6	50	,		
(CH ₂) ₁₀ , 2HBr	52	700	700	0 1 0 05 0 025	To	xic + -	5/7 1/7	70 15	10 5		
CH ₃ (C ₈ H ₁₇ NH CH ₂ CH-) ₂ (CH ₂) ₅ , 2HBr	53	1400	700	0 2 0 1	To +	xic +	3/5	60	10-5		
CH ₃ (C ₈ H ₁₇ NH CH ₂ CH-) ₂ (CH ₂) ₆ , 2HBr	54	>2000	1400	02		_	1/7	15	10-5	-	
$\left(\begin{array}{c} \text{CH}_{2}\text{-CH}_{2} \\ \text{CH}_{2}\text{-CH}_{2} \end{array}\right)$ CH NH- $\left(\begin{array}{c} \text{CH}_{2}\text{)}_{8}, \text{ 2HCl} \\ \text{2} \end{array}\right)$	55	390	200	0 5 0 2 0 1	То + +	xic + +	3/4 4/5	75 80	10-4	10-4	2,3
,, (CH ₂) ₁₀ , 2HCl	56	850	400	02			1/6	15	10 ⁻⁵		2,3,6

TABLE I-continued

		App	rox	Amoebicidal tests							
	Ref	(mg			i	ı vivo			in vi	tro	Other
Substance	No	Oral	Sub- cut	% diet		ecal lition	Rats	%	Amoe- bicidal conc	Bact -cidal conc	tests
(1)	(2)	⁻ (3)	(4)	(5)	Wall (6)	Conts (7)	(8)	(9)	(10)	(11)	(12)
$ \begin{array}{c c} \hline \left(O & CH_2-CH_2 \\ CH_2-CH_2 & N-\right)_{1} (CH_2)_{10}, 2HCI \end{array} $	57	1600	180	0 5 0 2	-	_	2/4 2/8	50 25		_	
((C ₂ H ₅) ₂ N(CH ₂) ₂ NH-) ₂ (CH ₂) ₁₀ , 4HBr	58	1200	300	02	-		1/6	15	_	_	(6)
(C ₈ H ₁₇ NH-) ₂ (CH ₂) ₅ , 2HBr	59	700	350	0 5 0 2 0 1 0 05	+ + + + + -	++++++	Toxic 5/7 1/8 0/8	70 10 0	10-5	-	
(C ₃ H ₇ NH-) ₂ (CH ₂) ₂ , 2HBr	60	374	2000	0 5 0 2			1/4 1/4	25 25	 	-	1,3
(C ₇ H ₁₆ NH-) ₂ (CH ₂) ₃ , 2HBr	61	10	275	0 2 0 1		_	6/11 3/6	55 50	10 5	10 4	6
(C ₁₃ H ₂₇ NH-) ₂ (CH ₂) ₃ , 2C ₃ H ₆ O ₃	62	>2000	>2000	0 5 0 2 0 1		_ _ _	4/7 0/5 1/5	60 0 20	-		6
NH (CH ₂) ₁₀ NH ₂ , 2HCi	63	1500	250	01	-		1/6	15		_	
$((C_4H_9)_2N-)_2(CH_2)_{10}, 2C_4H_6O_4$	64	500	950	0.5	_	_	3/6	50	10 4	_	
((C ₅ H ₁₁) ₂ N-)_(CH ₂) ₁₀ , 2HCl	65			05 02 01	+ +	+ +	3/7 7/14 3/12		10 5	-	
CH ₃ O (CH ₂) ₂ N (CH ₂ -CH ₂ -CH ₂ ,HBr (CH ₂ -CH ₂ -CH ₂ -CH ₂	66	70	470	01	-	_	1/4	25	_		1,3

noted that in some instances the oral toxicity of a substance is greater than the subcutaneous toxicity. This rather unusual finding is explained by the fact that most of the compounds are irritant, and whereas a localized necrosis is produced by subcutaneous injection, oral administration may result in severe and fatal damage to the walls of the alimentary tract, with secondary infection

In the amoebicidal tests, the proportion of rats protected from infection has been expressed as a percentage, which facilitates comparison of the effects of one drug with another. As we are very conscious of the fact that percentages calculated from small numbers of animals have little meaning, the actual numbers of animals used are

recorded, so that the true significance of the percentage figures may be apparent It should be remembered that control animals may show an infection rate as low as 80 per cent, and a figure of 20 per cent protection may not indicate activity Control animals were used in every of a drug The numbers of animals used were insufficient to give accurate comparisons between drugs, but were adequate to indicate whether or not a drug was likely to be of value in the treatment of The effect upon the macroscopic amoebiasis appearance of the caecal wall and contents has been recorded here merely as a significant improvement (+) or no significant improvement (-), detailed figures are not given

DISCUSSION OF THE RESULTS IN TABLE I

Phenylalkylamine series

All the members of the series of $bis(\beta-3)$ 4-dimethoxyphenylethylamino)alkanes (Nos 1 to 6) showed some activity m vivo but there was no significant difference between them. The improvement of the caecum produced by the compound containing 10 methylene groups suggests that it may have a slightly higher potency than the rest, and this is confirmed by the m vitro test

When only one methoxy-group was present as in Nos 7 and 8, the activity was reduced, and a similar effect was apparent in compounds 9 and 10 which contain free hydroxy-groups in the para positions of the benzene nuclei

The introduction of iodine atoms into the benzene nucleus as in Nos 11 and 12 gave rise to insoluble compounds with no increase in activity, although in this case the hexane- was more active than the decane-derivative

The β -p-chlorophenylethylamino derivatives 13, 14, and 15 had some activity, the corresponding o-chloro analogues (Nos 17 and 19) were rather more active both m vivo and m vitro, but were also more toxic

In the $bis(\beta$ -phenylethylamino)alkane series (Nos 22-27) all the compounds showed activity of a high order *in vitro*. The greatest *in vivo* activity was found in the higher members of the series. The effect of varying the length of the link between the phenyl and the amino groups is shown in Nos 28 to 33. The optimum length seems to be a chain of two methylene groups. An increase in activity in the bis(phenylmethylamino)alkane series was found when a methylenedioxy-group was introduced into the 3. 4- positions (No. 34), but a single *p*-methoxy or *p*-dimethylamino group was deleterious (Nos. 36 and 37)

Alkylamine series

The complete "opening out" of the emetine molecule leads to two n-octylamine residues connected through the N atoms by a chain of methylene groups. Owing to the ready availability of n-heptylamine via heptaldoxime we first studied the action of bis(n-heptylamino) alkanes. All these compounds (Nos 38 to 44) had $in\ vitro$ activities of the same order as the $bis(\beta$ -phenylethylamino) alkane series, but in addition had bactericidal action, which probably complicated the results of the $in\ vitro$ tests. In vivo these compounds were perhaps slightly more active than the phenylethylamino derivatives, but the results at low dose levels were erratic

Alteration of the length of the alkyl chain as in Nos 45 to 49 had little effect upon the activity, but the higher members had greater toxic effects when given in the diet than the lower members

Branching the side-chain as in No 50, 1 10-bis(2'-ethyl-n-hexylamino)decane and in No 51, 1 10-bis(triallylcarbinamino) decane, where the chain is both branched and unsaturated, did not increase the *in vivo* activity, but the longer, branched unsaturated compound, bis-geranyl-amino)decane (No 52) again showed a high *in vivo* activity, accompanied however by an increased toxicity

The introduction of methyl groups into the methylene chain connecting the two basic radicals (Nos 53 and 54) had little effect

When the alkyl chain was replaced by a cyclohexyl radical (Nos 55 and 56) the activity was not appreciably affected, but replacement of the alkylamino residues by tertiary basic groups as in 1 10-NN'-dimorpholyldecane (No 57) resulted in complete loss of activity both in vitro and in vivo. The introduction of a basic side chain as in 1 10-bis(β -diethylaminoethylamino)decane (No 58) had a similar effect

Reduction in the number of methylene groups connecting the two secondary amino groups to five or three, even when the molecular weight was increased by the introduction of longer alkyl groups, caused almost complete loss of activity (Nos 59 to 62)

The primary diamine, 1 10-diaminodecane (No 63), was completely inactive and the difertiary bases, 1 10-bis(dibutylamino)- and 1 10-bis(diamylamino)decane (Pyman, 1937, Nos 64 and 65), were considerably less active than the disecondary bases of about the same molecular weight (cf Nos 44 and 47)

The cyclic tertiary amine No 66, obtained as a by-product, was inactive

The effect of solubility

The activities of the sparingly soluble hydrobromides and the readily soluble lactates were not significantly different (Nos 1, 2, 22, 23, 38, 39, 41, 42, 46, 47) Nos 15,17, and 19 have been converted into insoluble bismuth iodides of variable composition, Nos 16, 18, 20 and 21, these were less toxic, but the activities were no greater than those of equivalent amounts of the parent compounds, except No 16, which was slightly more active than No 15

None of the compounds is comparable with emetine in amoebicidal activity. Emetine protects most of the rats at a concentration in the diet of

0 001 per cent, whereas the best of the synthetic compounds are practically useless at concentrations lower than 0 1 per cent

Although many members of the series have acute toxicities which are less than that of emetine, they are very irritant substances and it is unlikely that any would be suitable for clinical trial

CHEMICAL SECTION

Two general methods were used for the preparation of the secondary diamines, (a) an α , ω -alkylene dihalide was treated with a primary amine, or (b) an α,ω -alkylene diamine with an alkyl halide The reactions were carried out in a variety of solvents—alcohol, amyl alcohol, ether, acetone, benzene, toluene or xylene - or without solvent, and for varying lengths of time choice of method was usually determined by the availability of the starting materials In general the first method gave better yields, but, using alkylene dihalides containing 5 or 6 carbon atoms, there was a tendency for ring closure to take place with formation of N-alkyl(aralkyl)piperidines or -hexamethyleneimines The bis(phenylmethylamino) alkanes were most conveniently prepared ua the anils

1 6-Bis(β-3' 4'-dimethoxyphenylethylamino)hexane (No 1) β-3 4-Dimethoxyphenylethylamine (7 24 g), 1 6-dibromohexane (4 88 g) and xylene (20 cc) were allowed to stand for 24 hours and then heated to 115° for 30 mins. A crystalline solid separated on cooling, after recrystallization from alcohol glistening platelets of 1 6-bis(β-3' 4'-dimethoxyphenylethylamino)hexane dihydrobromide, mp 260-262°, were obtained in 29% yield (Found C, 51 5, H, 7 0, N, 4 6, Br, 26 6 C₂₆H₄₀O₄N, 2HBr requires C, 51 5, H, 7 0, N, 4 6, Br, 26 4%) The dilactate (No 2) forms plates from acetone, mp 120° (Found C, 61 4, H, 8 4 C₂₆H₄₀O₄N₂,2C₃H₆O₃ requires C, 61 5, H, 8 4%)

1 7-Bis(β -3' 4'-dimethoxyphenylethylamino)heptane (No 3) was obtained similarly in 24% yield as dihydrobromide from 1 7-dibromoheptane and β -3 4-dimethoxyphenylethylamine It forms leaflets from alcohol, mp 240° (Found N, 47, Br, 258 $C_{27}H_{42}O_4N_2$, 2HBr requires N, 45, Br, 258%) The dihydrochloride forms needles from alcohol, mp 247° (Found C, 607, H 82 C_2 - H_4 - O_4N_2 ,2HCl requires C, 610, H, 83%)

1 8-Bis(β-3' 4'-dimethox yphenylethylamino) octane (No 4) The dihydrobronide, mp 251°, was obtained similarly from 1 8-dibromo-octane and the amine in 40% yield (Found N, 48, Br, 255 $C_{28}H_{44}O_4N_2$,2HBr requires N, 44, Br 252%) The dihydrochloride forms needles from alcohol, mp 245-247° (Found C, 618, H, 86, N, 53 Cl, 124 $C_{18}H_{44}O_4N_2$,2HCl requires C, 616, H, 85, N, 51, Cl, 130%)

1 9 Bi·(β-3' 4'-dimethox) phen) lethylamino) nonane (No 5) The dilivdrobromide, obtained similarly from 1 9-dibromononane in 32% yield, forms leaflets from alcohol, mp 248° (Found N, 46, Br, 247 C-3H₄₆O₄N, 2HBr requires N, 43, Br, 2465%) The dilip drochloride forms needles from alcohol, mp 251° (Found Cl, 128 C-3H₄₆O₄N-2HCl requires Cl 127%)

1 10-Bis(β -3' 4'-dimethoxyphenylethylamino)decane (No 6) The dihydrobromide, leaflets from alcohol, mp 251°, was obtained similarly in 45% yield from 1 10-dibromodecane and homoveratrylamine (Found N, 45, Br, 242 $C_{50}H_{48}O_4N_2$,2HBr requires N, 42, Br, 241%) The dihydrochloride forms needles from alcohol, mp 244–245° (Found C, 629, H, 89 $C_{50}H_{48}O_4N_2$,2HCl requires C, 628, H, 88%)

1 6-Bis(β-p-methoxyphenylethylamino)hexane (No 7) β -p-Methoxyphenylethylamine (15 1 g), alcohol (25 ∞) and 1 6-dibromohexane (6 1 g) were heated under reflux for 24 hours and 2N-alcoholic HBr (25 cc) added The crystalline precipitate was filtered off and boiled with alcohol to remove p-methoxyphenylethylamine hydrobromide The residue, recrystallized from a large volume of alcohol, deposited 1 6-bis(β-p-methox) phenylethylammo)hexane dihydrobromide in crystals mp 308° (Found C, 528, H, 74, N, 51, Br, 291 C₂₁H₃₆O₂N₂, 2HBr requires C, 52 75, H, 70, N, 51, Br, 29 25%) The dipicrate forms yellow crystals from alcohol, mp (Found C, 513, H, 51, N, 135 C₄H₃₆O N, 2C₆H₃O N₃ requires C, 51 3, H, 50, N, 13 3%) The bases regenerated from the original mother-liquors were dissolved in alcohol and on addition of a hot alcoholic solution of picric acid deposited an oil, the clear decanted liquor on standing deposited orange crystals of β pmethoxyphenylethylamine picrate mixed with yellow crystals of N-β-p-methoxyphenylethylhexamethyleneimine picrate These were readily separated by hot benzene in which the former is insoluble. The latter formed yellow crystals from benzene, m p 151°, yield 37% (Found C, 547, H, 60, N, 122 $C_{1b}H_{23}ON$, $C_6H_2O_7N_3$ requires C, 545, H, 57, N, 121%) The hydrobromide (No 66), colourless crystals from alcohol, melts at 196° (Found C, 573, H, 79, N, 44, Br, 256 C₁₅H₂ON, HBr requires C, 573, H, 79, N, 45, Br, 254%) The ring structure was confirmed by failure of the base to yield an acetyl derivative and by the formation of a methodide, m p 132° (Found C, 51.1, H, 71, N, 39, OMe, 83, NMe 75 C₁₅H₂₃ON, CH₃I requires C, 512, H, 70, N, 37, OMe, 83, NMe, 77%)

1 10-Bis(β-p-methoxvphenylethylamino)decane (No 8) The dihydrobromide was obtained in 53% yield in a similar manner to that just described but omitting the treatment with picric acid It separates from a large volume of alcohol in crystals, mp 304° (decomp) (Found N, 49, Br, 267 C₂₈H₁₄O₂N₂,2HBr requires N, 465, Br, 265%)

1 6-Bis(β-p-hydrox) phenylethylamino) hexane (No 9) This was prepared by heating the methoxy derivative (No 7) with 8 times its weight of hydrochloric acid (d, 12) in a sealed tube at 170° for 6 hours The dihydrochloride separated from water in silky needles, mp 255-257° (Found C, 612, H, 79, N, 63, Cl, 168 C₂₂H₂₂O₂N₂,2HCl requires C, 615, H, 80, N, 65, Cl, 165%)

1 10-Bis(β-p-hydroxyphenylethylamino)decane (No 10)
The corresponding methoxy-compound (No 8) heated in a sealed tube with hydrochloric acid (d, 1 2) furnished 1 10-bis(β-p-hydroxyphenylethylamino)decane dihydrochloride in needles from water mp 238° (decomp)

(Found C, 64 8, H, 8 7 N 5 4, Cl, 14 7 $C_{26}H_{40}O_2N_2$, 2HCl requires C, 64 3, H, 8 7, N, 5 8, Cl, 14 6%)

1 6-Bis(β -3'-iodo-4'-hisdroxyphenslethslamino)hexane (No 11) To a stirred solution of the hydroxy-compound (No 9) (2 0 g) in methyl alcohol (120 cc) 10% aquicous ammonia (4 cc.) was added followed by iodine (2 4 g) in 50% potassium iodide solution (9 6 g). The precipitate was collected, washed in turn with methanol, water and methanol. The base formed an almost white powder, mp 175°, yield 94% (Found I, 42 4 $C_{22}H_{30}O_2N_2I_2$ requires I, 41 7%). The iodine atoms are assumed to be in the 3'-positions

1 10-Bis(%-3'-iodo-4'-list droxyphen) leths lamino) decane (No 12) This was prepared in 93% yield from No 10 in a similar way. The base melted at 169° (decomp.) (Found C, 469, H, 62, N, 45, I, 403 $C_rH_{34}O_2N_1I_2$ requires C, 470, H, 58 N, 42, I 382%.) The rodine atoms are assumed to be in the 3'-positions

8-p-Chloropheny lethy lanune Dry ammonia was passed for 3-4 hours through fused p-chlorophenylpropionic acid at 180°. The powdered product was freed from a little unchanged acid by extraction with aqueous ammonia and the insoluble p chloropheny lpropionamide recrystallized from benzene It formed tetragonal plates, mp 133°, yield 82% (Found C, 593, H, 58, N, 79 Cl, 193 C.H₁₀ONCl requires C, 589, H, 55, N, 76, Cl, 193%) It was converted into p-chlorophenylethylamine, b p 129-135/24 mm, by the method of McRae and Vining (1932) The picrate forms light orange-coloured prisms from alcohol, m.p. 212° (Found N, 148 Cl, 93 C₈H₁₀NCl, C₆H₂O-N₃ requires N, 146, Cl, 9 2%) The hydrobronude separates from alcohol in colourless crystals, m p 237° (Found C, 406, H, 50, N, 57 C₄H₁₀NCl, HBr requires C, 406, H, 47, N, 59%)

β-o-Chlorophenylethylamine This base was prepared in 67% yield from β-o-chloropropionamide in a similar manner. It is a colourless liquid, b p $128^{\circ}/23-26$ mm The picrate forms yellow crystals from alcohol, m p 187° (Found C, 43 7, H, 3 9, Cl, 9 2 C₈H₁₀NCl,C₆H₃O₇N₃ requires C, 43 7, H, 3 4, Cl, 9 2%) The hydrobromide, which is very soluble in alcohol, separates from a concentrated solution as a dihydrate, m p 92° with previous sintering (Found loss at 50° in a vacuum 122 C₈H₁₀NCl,2H₂O requires loss 132%) The anhydrous salt has m p 190° (Found C, 409, H, 49, N, 61, Cl, 147, Br, 332 C₈H₁₀NCl,HBr requires C, 406, H, 47, N, 59, Cl, 150, Br, 338%)

1 6-Bis(β-p-chlorophenylethylamino)hexane (No 13) β-p-Chlorophenylethylamine (4 mols) in alcohol was heated under reflux for 24 hours with 1 6-dibromohexane (1 mol) The product was neutralized with 2N-alcoholic hydrobromic acid and after standing the crystalline deposit was filtered off and recrystallized to give 1 6-bis(β-p-chlorophenylethylamino)hexane dihydrobromide in colourless crystals, mp 301° (decomp), yield 29% (Found N, 5 2, Cl, 12 5, Br, 28 1 $C_{22}H_{30}N_2Cl_2$,2HBr requires N, 5 1, Cl, 12 8, Br, 28 8%) The dipicrate separated from alcohol in yellow crystals, mp 219° (Found C, 48 5, H, 48, N, 12 9, Cl, 8 0 $C_{22}H_{30}N_2Cl_2$,2Ce $_{6}H_{30}O_{7}N_{3}$ requires C, 48 0, H, 4 3,

N, 13 2, Cl, 8 3%) The bases liberated from the mother-liquors from the above hydrobromide were converted to picrates from which N-β-p-chlorophenyl-ethylhexamethyler cumine picrate was obtained by extraction with benzene. It separated from this solvent in yellow needles, mp 181° in a yield of 32% (Found C, 51 8, H, 54, N, 12 1, Cl, 7 5 C₁₄H-₀NCl,C₆H₃O-N₃ requires C, 51 45, H, 50, N, 12 0, Cl, 76%) The Indrobromide forms plates from alcohol, mp 263° (decomp) (Found C, 52 8, H, 6 7, N, 4 6, Cl, 11 1 Br, 25 1 C₁₄H-₀NCl,HBr requires C, 52 75, H, 6 6, Cl, 11 1, Br, 25 1%)

1 8-Bis(β-p-chlorophenvlethvlamino) octane (No 14) The dihi drobromide was obtained in 44% yield by the above process using 1 8-dibromo-octane and p-chlorophenylethylamine. It is very sparingly soluble in alcohol from which it is deposited in crystals, m. p. 291° (decomp.) (Found. C., 494, H., 65, N., 49, Cl., 117, Br., 264 C.₄H₃₄N₂Cl₂,2HBr requires C., 494, H., 62, N., 48, Cl., 122 Br., 274%) The dipicrate forms yellow crystals from alcohol, m.p. 183° (Found. C., 493, H., 46, N., 126 C₂₁H₃₄N₂Cl₂,2C₆H₃O₇N₃ requires C., 492, H., 46, N., 127%)

1 10-Bis(β-p-chlorophenylethylamino)decane (No 15) The diliydrobromide was obtained in 53% yield from 1 10-dibromodecane and the appropriate amine in crystals from alcohol, m p 290° (decomp) C, 513, H, 66, N, 46 C₂₆H₃₈N₂Cl₂,2HBr requires C, 51 1, H, 66 N, 46%) The dipicrate forms yellow needles from alcohol, mp 170° (Found C, 503, H, 50, N, 120 $C_{27}H_{38}N_2Cl_2C_6H_3O_7N_3$ requires C, 50 3, H, 49, N, 12 35%) The dilactate separates from alcohol in crystals, mp 160° (Found N, 46, $C_{26}H_{38}N_2Cl_2,2C_3H_6O_3$ requires N, 445, Cl, 119 Cl, 11 3%) The bismuth iodide (No 16), formed by addition of excess of potassium bismuth iodide to a solution of the lactate in water forms an insoluble red powder approximating in composition to B,2HI,BiI₃ (Found base 25 3 B,2HI,Bil₃ requires base, 23 8%)

1 8-Bis(β-o-chlorophenylethylamino)octane (No 17) The dihy drobromide was obtained in 38% yield by heating β-o-chlorophenylethylamine and 1 8-dibromo-octane in alcoholic solution. It is very sparingly soluble in alcohol from which it separates in crystals, mp 266° (decomp) (Found C, 49 8, H, 6 4, N, 4 7 C₂₄H₃₄N₂Cl₂,2HBr requires C, 49 4, H, 6 2, N, 4 8%) The dipicrate forms yellow crystals from alcohol, mp 154° (Found C, 49 3, H, 4 7, N, 13 1 C₂₄H₃₄N₂Cl₂,2C₆H₃O₇N₃ requires C, 49 2, H, 4 6, N, 12 7%) The dilactate is very soluble in alcohol and crystallizes on addition of acetone, mp 171° (Found N, 4 6, Cl, 11 8 C₂₄H₃₄N₂Cl₂,2C₃H₆O₃ requires N, 4 65, Cl, 11 8%) The bismuth iodide (No 18) prepared in the usual manner from the lactate formed an insoluble red powder containing 26% of base

1 10-Bis(β-o-chlorophenylethylamino)decane (No 19) The dihydrobromide was obtained in 45% yield from 1 10-dibromodecane and the appropriate amine, it separates from much alcohol in crystals, mp 262° (decomp) (Found C, 51 4, H, 6 9, N, 4 3, Cl, 11 7, Br, 26 4 C₂₆H₃₈N₂Cl₂, 2HBr requires C, 51 1; H, 6 6,

N, 46, Cl, 116, Br, 2415%) The dipicrate forms yellow crystals from alcohol, mp 121° (Found N, 124, Cl, 75, C₂₆H₃₈N₂Cl₂,2C₆H₃O N₃ requires N, 1235, Cl, 78%) The dilactate (No 19) is deposited from hot alcohol in crystals, mp 131° (Found C, 611, H, 81, N, 47 C₂₆H₃₈N₂Cl₂,2C₃H₆O₃ requires C, 610, H, 80, N, 445%) Two different bismuth iodides were obtained by the addition of varying amounts of potassium bismuth iodide to an aqueous solution of the lactate Preparation A (No 20) contained 36 6% of base and B (No 21) 22 9% of base These correspond approximately to compounds of the composition B,2HI,BiI₃ and B,2HI,2BiI₃ which require 34 65% and 23 8% of base respectively

1 6-Bis(β-phenylethylamino)hexane (No 22) β-Phenylethylamine (10 g) and 1 6-dibromohexane (10 16 g) in benzene (30 cc) were heated under reflux for 2 hours. After cooling 1 6-bis(β-phenylethylamino)hexane dihydrobromide was collected and recrystallized from alcohol. It forms plates, mp 314–317°, yield 12 5% (Found C, 54 7, H, 7 2, N 6 1, Br, 32 55 C₂₂H₃₂N₂,2HBr requires C, 54 3, H, 7 05, N, 5 8, Br, 32 9%) The dilactate (No 23) separates from alcohol-acetone in prisms, mp 155–157° (Found C, 66 6, H, 8 95 C₂₂H₃₂N₂,2C₃H₆O₃ requires C, 66 7, H, 8 8%)

1 7-Bis(β -phenylethylamino)heptane (No 24) The dihvdrobromide was obtained in 24% yield from 1 7-dibromoheptane and β -phenylethylamine by the above method It forms plates from alcohol, mp 313° (Found C, 55 2, H, 7 4 $C_{23}H_{34}N_2$,2HBr requires C, 55 2, H, 7 2%) The dilactate separates from acetone in crystals, mp 150-152° (Found C, 67 3, H, 9 1 $C_{23}H_{34}N_2$,2 $C_3H_6O_3$ requires C, 67 2, H, 8 9%)

1 8-Bis(β-phenylethylamino)octane (No 25) 1 8-Diamino octane (4 32 g), obtained by the action of hydrazoic acid on sebacic acid, was heated on a water bath for 15 mins with β-phenylethyl bromide (11 1 g) and benzene (10 cc) The solid which separated was collected and crystallized from alcohol The hydrobromide was thus obtained in leaflets, mp 312°, yield 27% (Found C, 56 4, H, 7 6 C₁₄H₃₄N₂,2HBr requires C, 56 0, H, 7 45%) The dilactate forms needles, mp 150-151° (Found C, 67 8, H, 9 1 C₋₁H₃₆N₂,2C₃H₆O₃ requires C, 67 6, H, 9 1%)

1 9-Bιs(β-pheny lethy lamuno) nonane (No 26) β-Phenylethylamune (12 1 g) and 1 9-dibromononane (14 3 g) dissolved in benzene (25 cc) were left to stand at room temperature for 48 hours. The crystalline solid which separated was recrystallized from alcohol to give the dihydrobromide, mp 300–302°, in 21 6% yield (Found C, 56 8, H, 8 05 $C_{15}H_{18}N_2$, 2HBr requires C, 56 8, H, 7 7%) The dilactate forms clusters of needles from alcohol-acetone, mp 134–136° (Found C, 68 3, H, 9 4 $C_{25}H_{38}N$, 2C₃H_rO₃ requires C, 68 1, H, 9 2%)

1 10-Bis(β -phen) leth lamino) decane (No 27) β -Phenylethylamine and 1 10-dibromodecane heated for 2 hours under reflux in benzene solution furnished the dihr drobromide in 13.5% yield after crystallization from alcohol, mp 302° - 304° (Found C, 57.7, H, 7.9

 $C_{26}H_{40}N_2$,2HBr requires C, 57 6, H, 7 8%) The dilactate forms needles from alcohol-acetone, m p 146° (Found C, 68 7, H, 9 1 $C_{26}H_{40}N_2$,2 $C_3H_6O_3$ requires C, 68 5, H, 9 35%)

1 8-Bis(γ -phenylpropylamıno)octane (No 28) The dihydrobromide was obtained in 37% yield by heating γ -phenylpropylamine (4 mols) with 1 8-dibromo octane (1 mol) in alcoholic solution for 24 hours. It forms crystals sparingly soluble in alcohol, mp 284° (decomp) (Found C, 57 5, H, 8 2, N, 5 1, Br, 29 9 $C_{26}H_{40}N_2$,2HBr requires C, 57 6, H, 7 8, N, 5.2, Br, 29 5%) The dipicrate gave yellow crystals from alcohol, mp 153° (Found C, 54 3, H, 5 75, N, 13 7 $C_{26}H_{40}N_2$,2 $C_6H_3O_7N_3$ requires C, 54 2, H, 5 5, N, 13 4%) The dilactate crystallizes from alcohol-acetone, mp 147° (Found C, 68 5, H, 9 4, N, 5 5 $C_{26}H_{40}N_2$,2 $C_3H_6O_3$ requires C, 68 5, H, 9 35, N, 50%)

1 10-Bis(γ phenylpropylamino)decane (No 29) The dihvdrvbronude, obtained in a similar manner in 50% yield from 1 10-dibromodecane and the amine, is sparingly soluble in alcohol and forms crystals, m p 283° (Found C, 59 1, H, 8 2, N, 4 3, Br, 28 4 C₂₈H₄₄N₂, 2HBr requires C, 58 9, H, 8 1, N, 4 9, Br, 28 0%) The dilactate separates from alcohol-acetone in crystals, m p 119° (Found N, 4 8 C₂₈H₄₄N₂,2C₃H₆O₃ requires N, 4 8%)

1 8-Bis(δ-phenylbutylamino)octane (No 30) The dihydrobronide was obtained similarly in 32% yield from δ-phenylbutylamine and 1 8-dibromo-octane It cry stallizes from alcohol in needles, mp 281° (decomp) (Found C, 58 3, H, 8 0, N, 5 3, Br, 28 9 C₈H_HN, 2HBr requires C, 58 9, H, 8 1, N, 4 9, Br 28 0%) The dilactate crystallizes on addition of acetone to a concentrated alcoholic solution, mp 149° (Found C, 69 5, H, 9 4, N, 5 0 C₂₈H_HN₂,2C₃H₆O₃ requires C, 69 35, H, 9 6, N, 4 8%)

1 10 Bis(δ-phenylbutylanino)decane (No 31) The dihydrobronude was obtained in 43% yield from δ-phenyl butylamine and 1 10 dibromodecane It is sparingly soluble in alcohol, mp 282° (decomp) (Found C, 60 4, H, 8 2, N, 4 8, Br, 26 9 C₃₀H₄₈N₂,2HBr requires C, 60 2 H, 8 4, N, 4 7, Br, 26 7%) The dilactate crystallized from alcohol, mp 125° (Found C, 69 8, H, 9 4, N, 4 8 C₃₀H₄₈N₂,2C₃H₆O₃ requires C, 70 1, H, 9 8, N, 4 5%)

1 8-Bis(ε-phenylamylamino)octane (No 32) The dihydrobramide was obtained in 73% yield from ε phenylamylamine and 1 8-dibronio-octane. It is fairly soluble in hot alcohol and separates in crystals m p 258° (Found C, 60 5, H, 8 0, N, 4 5 C₃₀H₁₈N, 2HBr requires C, 60 2, H, 8 4, N, 4 7%) The dilactate forms six-sided plates from alcohol-acetone, m p 117° (Found C, 70 0, H, 9 8, N, 4 7 C₃₀H₄₈N₂,2C₃H₄O₃ requires C, 70 1, H, 9 8, N, 4 5%)

1 8-Bis(phenvlmethylamino)octane (No 33) 1 8 Diamino-octane (29 g) was heated under reflux with benzaldehyde (45 g) for 1 hour to yield 1 8-bis-(benzvlideneamino)octane which after distillation at 220-230/0 1 mm crystallized on cooling, mp 30-31° (Found C, 82 8, H, 8 8 C₂₂H₂₈N₂ requires C, 82 5, H, 8 8%) The anil was reduced with hydrogen (PtO₂

catalyst) in alcoholic solution. On addition of hydrochloric acid (d 12) to the filtered solution 1 8-bis-(phenylmethylamino)octane dihydrochloride was obtained in p 276-280° after crystallization from alcohol. (Found C 66 4, H, 8 5 Cl, 17 9 C₂₂H₂₂N₂ 2HCl requires C, 66 5, H 8 6, Cl, 17 9%) The base formed crystals, in p 37-38 5° (Found C, 81 1, H, 9 8, N, 8 5 C₂₄H₃₂N₂ requires C, 81 4, H, 9 9, N, 8 6%)

1 8-Bis(3' 4'-methylenedioxyphenylmethylamino)octane (No 34) By a similar procedure, piperonal was
converted to 1 8-bis(3' 4'-methylenedioxybenzylideneamino)octane mp 111-112° from alcohol (Found
C, 70 8, H, 7 2 C₂₁H₂O₄N₂ requires C, 70 6, H, 6 9%)
Catalytic reduction gave the base, leaflets from alcohol,
mp 69-70° (Found C, 69 7, H, 8 1, N, 6 6
C₂₄H₂₂O₄N₂ requires C, 69 9, H, 7 8, N, 6 8%) The
dihydrochloride melted at ca 274° (Found C, 59 2,
H, 6 9 C₂₁H₂₂O₄N₂,2HCl requires C, 59 4, H, 7 1%)

1 8-Bis(3' 4'-dimethoxyphenylmethylamino)octane (No 35) Veratric aldehyde was converted similarly into 1 8-bis(3' 4'-dimethoxyberzylideneamino)ociane, leaflets from alcohol, mp 108 5-109 5° (Found C, 71 2, H, 8 5 C₂₆H₃₆O₄N₂ requires C, 70 9, H, 8 2%)

The base, obtained by catalytic reduction of the anil, formed leaflets from alcohol, mp 83° (Found C, 699, H, 90 $C_{26}H_{10}O_4N_2$ requires C, 702, H, 91%) The diln drochloride crystallized from alcohol, mp 231–232° (decomp) (Found C 605, H, 81, Cl, 140 $C_{26}H_{40}O_4N_2$,2HCl requires C, 604, H, 82, Cl, 137%)

1 8-Bis(p-methox) pheny limethylamino) octane (No 36) p-Methoxybenzaldehyde with 1 8-diamino-octane yielded 1 8-bis(p-methox) benzy lideneamino) octane, leaflets from alcohol, mp 64-65° (Found C, 747, H 83, N, 74 $C_{24}H_{32}O_2N_2$ requires C, 757, H, 85, N, 74%) Reduction of the anil gave the base, mp 605-615°, after crystallization from light petroleum (Found C, 747, H, 94, N, 71 $C_{21}H_{36}O_2N_2$ requires C, 750, H, 94, N, 73%) The dihydrochloride melted at 275-276° (Found C, 628, H, 83 $C_{24}H_{36}O_2N_2$,2HCl requires C, 630, H, 84%)

1 8-Bis(p-dimethylaminophenylmethylamino)octane (No 37) p-Dimethylaminobenzaldehyde and 1 8-diamino-octane treated in the usual manner gave 1 8-bis(p-dimethylaminobenzylideneamino)octane which crystallized from alcohol in yellow leaflets, mp 105-106° (Found C, 75 8, H, 9 3 C26H36N4 requires C, 76 8, H, 9 4%) Catalytic reduction furnished the base, long, fibrous needles from light petroleum, mp 65-66° (Found C, 75 7, H, 10 2, N, 13 2 C26H42N4 requires C 76 1, H, 10 3, N, 13 65%) The tetrahydrochloride formed a felted crystalline mass from alcohol, mp ca 214° (decomp) (Found N, 9 3, Cl, 23 8 C26H42N4, 4HCl requires N, 9 5, Cl, 23 9%)

1 6-Bis(n-heptylamino)hexane (No 38) n-Heptylamine (69 g) was mixed with 1 6-dibromohexane (73 g) and xylene (50 cc) After standing overnight the mixture was heated under reflux for $\frac{1}{2}$ hour On cooling, the dihydrobromide separated, it forms plates from alcohol, mp 329°, yield 34% (Found C, 50 6, H, 9 8 C₂₀H₄₄N₂,2HBr requires C, 50 6, H, 9 8%) The dihydrochloride crystallizes in shining plates from alcohol-

acetone, mp 340° (Found C, 628, H, 1195, N, 735, Cl, 185 $C_{20}H_{44}N_{2}$,2HCl requires C, 623, H, 120; N, 73, Cl, 184%) The dilactate (No 39) forms colourless needles from acetone, mp 105° (Found C, 630, H 112 $C_{20}H_{44}N_{2}$,2C₃H₆O₃ requires C, 634, H, 1145%)

1 7-Bis(n-heptylanimo)heptane (No 40) The dihydrobromide was obtained in a similar manner from heptylamine and I 7-dibromoheptane. It forms plates from alcohol, mp 318° (Found N, 58, Br, 328 $C_{21}H_{46}N_{2}$,2HBr requires N, 60, Br, 331%) The dilactate crystallizes from alcohol-acetone, mp 143–146° (Found C, 645, H, 113 $C_{21}H_{26}N_2$,2 $C_3H_6O_3$ requires C, 640, H, 112%)

1 8-Bis(n-heptvlamino) octane (No 41) n-Heptylamine (48 g) and 1 8-dibromo-octane (572 g) were heated under reflux with acetone (30 cc) until solid separated. After cooling, the dihydrobromide was collected. It forms leaflets from alcohol, mp 308°, yield 25% (Found N, 58, Br, 323 $C_{22}H_{48}N_{2}$,2HBr requires N, 545, Br, 318%) The dilactate (No 42) crystallizes from acetone in needles, mp 103–104° (Found C, 648, H, 115 $C_{22}H_{48}N_2$,2 $C_3H_6O_3$ requires C, 646, H, 116%)

1 9-Bis(n-heptylamino)nonane (No 43) The dihydrobronide was obtained from n-heptylamine and 1 9-dibromononane in 25% yield by heating in xylene for 7 hours It separates from alcohol in leaflets m p 305° (Found N, 56, Br, 314 $C_{23}H_{50}N_2$,2HBr requires N, 54, Br, 309%) The dilactate forms needles from acetone, m p 132–134° (Found C, 65 15, H, 12 1 $C_{23}H_{50}N_2$,2 $C_3H_6O_3$ requires C, 65 1, H, 11 7%)

1 10-Bis(n-heptylamino) decane (No 44) The dihydrobromide prepared similarly in 24% yield from n-heptylamine and 1 10-dibromodecane forms leaflets from alcohol, mp 320-322° (Found C, 54 55, H, 10 2, N, 5 5, Br, 30 2 $C_{24}H_{52}N_{2}$, 2HBr requires C, 54 3, H, 10 3, N, 5 3, Br, 30 1%) The dilactate, needles from dry alcohol-acetone, melts at 138-139° (Found C, 65 6, H, 11 8 $C_{24}H_{52}N_2$, 2 $C_3H_6O_3$ requires C, 65 7, H, 11 8%)

1 10-Bis(n-hexylamino)decane (No 45) The dihydrobromide was obtained in 45% yield from 1 10-dibromodecane and n-hexylamine (4 mols) by heating for 16 hours in dry alcohol It forms colourless needles, mp 315–317° (Found C, 530, H, 98, N, 60, Br, 319 $C_{22}H_{48}N_{2,2}HBr$ requires C, 526, H, 100, N, 56, Br, 318%) The dilactate separates from alcohol in feathery needles, mp 141–143°-c (Found C, 646, H, 116, N, 51 $C_{22}H_{48}N_{2,2}C_{3}H_{6}O_{3}$ requires C, 6455, H, 116, N, 54%)

1 8-Bis(n-octylamino)octane (No 46) The dihydrobromide, obtained in 42% yield from 1 8-dibromoctane and n-octylamine (4 mols) in boiling alcohol (20 hours), forms plates, mp 314° (Found C, 542, H, 92, N, 55 $C_{24}H_{52}N_2$,2HBr requires C, 543, H, 103, N, 53%) The dilactate (No 47) separates in plates from alcohol, mp 105-106° (Found C, 654, H, 115, N, 47 $C_{24}H_{62}N_2$,2 $C_3H_6O_3$ requires C, 657, H, 118, N, 51%)

- 1 8-Bis(n-nonylamnno)octane (No 48) The dihydrobromude was obtained similarly from n-nonylamine and 1 8-dibromo-octane in platelets, in p 310-312° (Found C, 56 4, H, 9 3, N, 4 95, Br, 28 8 C₂₆H₅₆N_{.,2}HBr requires C, 55 9, H, 10 5, N, 5 0, Br, 28 6%) The dilactate (No 48) forms short thin needles from dry alcohol-acetone, in p 109-111° (Found C, 66 4, H, 11 95, N, 4 5 C₂₆H₅₆N₂,2C₃H₆O₃ requires C, 66 6, H, 11 9, N, 4 9%)
- 1 10-Bis(n-undecylamino)decane (No 49) 1 10-Diaminodecane (4 3 g) in amyl alcohol (26 cc) was boiled under reflux and n-undecylbromide (11 8 g) added gradually during 6 hours. Heating was continued for a further 18 hours. On cooling the dihydrobromide separated as a mass of crystals which after recrystallization from isopropylalcohol and from alcohol formed feathery needles, mp 302-305°, yield 20% (Found C, 59 85, H, 11 05, N, 4 6, Br, 25 2 C₃₂H₀₈N, 2HBr requires C, 59 7, H, 11 0, N, 4 4, Br, 24 9%)
- 1 10-Bis(2'-ethyl-n-hexylamino)decane (No 50) Bromo-2-ethyl-n-hexane (193 g) and 1 10-diaminodecane (86 g) were heated under reflux in benzene (50 cc) for 15 hours After removing the benzene, the residue was heated for 15 minutes with 2% alcoholic sodium hydroxide, the alcohol distilled off and the bases extracted with ether The residue from the ether was extracted with hot ligroin (b p 90-120°), cooled, filtered from diaminodecane and then treated with dry HCI The diliydrochloride which separated formed fine needles from water, m p 128-132°, yield 10% (Found C, 663, H, 121, N, 60, Cl, 154 C26H86N2,2HCl, requires C, 665, H, 124, N, 60, Cl, 151%) The dihydrobromide crystallizes from alcohol in fine needles, mp 169-172° (Found C, 563, H, 105, N, 53, Br, 286 C₂₆H₅₆N₂,2HBr requires C, 559, H, 105, N, 50, Br, 28 6%)
- 1 10-Bis(triallylcarbinamino)decane (No 51) 1 10-Dibromodecane was heated in alcoholic solution with 7 mols of triallylcarbinamine (Henze, Allen and Leslie, 1943) for 24 hours The solvent was removed and the crystalline residue shaken with ether and sodium hydroxide The ether on evaporation left an oil from which excess of triallylcarbinamine was removed by distillation under reduced pressure at an oil bath temperature of 140° The residue was converted to dihydrochloride, colourless crystals from water, mp 246-251° (decomp) (Found C, 700, H, 105, N, 55, Cl, 135 $C_{30}H_{4}$ -N-,2HCl requires C, 7015, H, 106, N, 55, Cl, 13 8%) The dinitrate forms clusters of crystals from water, m p 190° (decomp) (Found C, 638, H, 985, N, 100 C₃₀H₅₂N₂,2HNO₃ requires C, 636, H, 96, N, 99%)
- 1 10-Bis(geranylamino)decane (No 52) 1 10-Dibromodecane was heated under reflux for 24 hours with 4 parts by weight of geranylamine Addition of ether precipitated a mixture of hydrobromides which on recrystallization from isopropylalcohol gave silky platelets of bis(geranylamino)decane diliy drobromide, m p 218° (Found C, 59 3, H, 9 4, N, 4 6, Br, 26 7 C₂₀H₅₆N₂, 2HBr requires C, 59 4, H, 9 6, N, 4 6, Br, 26 35%)

- 1 9-Bis(n-octylamino)-2 8-dimethylnonane (No 53) 2 8-Dimethyl-1 9-dibromononane (5 4 g) and n octylamine (17 78 g) in dry alcohol (30 cc) were heated under reflux for 16 hours, the solvent removed and the residue shaken with ether and alkali. After removing the ether, excess of octylamine was distilled off under reduced pressure (12 0 g b p 96-98°/45 mm) and the residue converted to dihydrobromide. After recrystallization from isopropyl alcohol the salt was obtained in 60% yield, m p 248-251° with previous sintering (Found C, 56 5, H, 10 35, N, 4 7 C₇₇H₅₈N₂,2HBr requires C, 56 6, H, 10 6, N, 4 9%)
- 1 10-Bis(n-octylamino)-2 9-dimethyldecane (No 54) The dihydrobromide was obtained in a similar manner from 2 9-dimethyl-1 10-dibromodecane, in 50% yield It forms fine needles from isopropyl alcohol, mp 242-244°, with previous sintering (Found C, 5765, H, 107, N, 505 C₈H₆₀N₂,2HBr requires C, 573, H, 107, N, 48%)
- 1 8-Bis(cyclohexylamino) octane (No 55) was obtained from 1 8-dibromo-octane and cyclohexylamine After removal of excess cyclohexylamine the base was distilled at about 200°/0 2 mm (yield 84%) After a second distillation the base crystallized on cooling, mp 27-28° (Found N, 89 C₁₀H₄₀N₂ requires N, 91%) The dihy drochloride forms colourless crystals from hot alcohol in which it is sparingly soluble, mp 284-285° (Found C, 630, H, 111, Cl, 186 C₁₀H₄₀N₂,2HCl requires C, 630, H, 111, Cl, 186%)
- 1 10-Bis(cyclohe vlamino) decane (No 56) was obtained in a similar manner from 1 10-dibromodecane in 95% yield The base boils at ca 205°/02 mm and crystallizes on cooling, mp 35-36 5° (Found C, 783, H, 131 C₂₂H₄₄N₂ requires C, 785, H, 132%) The dihydrochloride forms colourless crystals from alcohol, mp 322° (decomp) (Found C, 647, H, 113, Cl, 1745 C₂H₄₄N₂,2HCl requires C, 645, H, 113, Cl, 173%)
- 1 10-N N'-Dimorpholyldecane (No 57) 1 10 Dibromodecane (4 5 g) was heated under reflux for 12 hours with morpholine (5 22 g) and dry alcohol (15 cc) Some morpholine hydrochloride separated The mixture was made alkaline with NaOH and steam distilled to remove morpholine and alcohol The residue was extracted with ether and the base converted to dihydrochloride which separates from dry alcohol in needles, mp 240-242° (decomp) yield 84% (Found C, 56 15, H, 10.3 N, 74, Cl, 18 25 C₁₈H₃₀O₂N, 2HCl requires C, 56 1, H, 99, N, 73, Cl, 18 4%)
- 1 10-Bis(β-diethylaminoethylamino)decane (No 58) 1 10-Diaminodecane (8 2 g) in benzene (50 cc.) was mixed with freshly prepared β-diethylaminoethyl chloride and left overnight. Potassium carbonate (7 9 g) was added and the mixture heated under reflux for 20 hours. After removing the benzene the residue was shaken with ether and sodium hydroxide solution. The ether was dried and evaporated, and the residue distilled. A fraction b p 200-240°/6 mm was collected and converted to tetralin drobromide. It forms microscopic needles from amyl alcohol, m p 190-192° (3 5 g). (Found C, 37 9,

H, 80, N, 89, Br 456 $C_{20}H_{50}N_{4}$,4HBr requires C, 380, H, 78, N, 81, Br, 460%)

- 1 5-Bis(n-octvlanuno)pentane (No 59) Cadaverine (10 g) in absolute alcohol (5 cc) was heated under reflux for 7 5 hours with n-octyl bromide (3 8 g). The diln drobromide separated from alcohol in flat needles, mp 329-332° (decomp), yield 15% (Found Br, 33 6 $C_{21}H_{40}N_2$,2HBr requires Br, 32 7%) By heating 1 5-dibromopentane with n-octylamine only N-octylpiperidine, bp 136-8/18 mm was obtained. The hydrochloride had mp 189-191° (cf v Braun and Buchmann, 1931)
- 1 3-Bis(n-prop) lamino) propane (No 60) The diliv dro-bronide was obtained by adding 1 3-dibromopropane (20 2 g.) slowly during 2 5 hours to a boiling solution of *n*-prop) lamine (11 8 g) in benzene (20 cc) The mixture was heated for a further 4 hours, the hydrobromide filtered off and recrystallized from alcohol It forms plates, mp 304° (70 g) (Found N, 875, Br, 49 7 C₈H₂₂N₂,2HBr requires N, 875, Br, 500%)
- 1 3-Bis(n-hepti lamino) propane (No 61) n-Heptylamine (115 g) was mixed with 1 3-dibromopropane (101 g) in benzene (10 cc) The mixture developed heat, it was left to stand for several days, the mush of crystals filtered off and washed with water to remove heptylamine hydrochloride The residue of 1 3-bis(n-hepti lamino) propane dilit drobromide furnished platelets from alcohol, mp 320-322° (Found C 4725, H, 89, N, 675, Br, 3725 C₁₇H₁₈N, 2HBr requires C, 472, H, 93, N, 65, Br, 370%)
- 1 3-Bis(n-tridecylamino)propane (No 62) The dihydrobromide was obtained in 65% yield by heating 1 3-dibromopropane and n-tridecylamine in dry alcohol for 18 hours. After two crystallizations from alcohol it formed platelets, mp 304–307° (Found N, 48 $C_{29}H_{62}N_2$,2HBr requires N, 47%) The dilactate separates from alcohol in rosettes of stout needles, m.p 148–150°, very sparingly soluble in water (Found C, 680, H, 120, N, 48 $C_{29}H_{62}N_2$,2 $C_3H_6O_3$ requires C, 679, H, 1205, N, 45%)
- 1 10-Bis(di-n-but) lamino) decane (No 64) This was obtained by the method of BP 433,086 The acid succinate separates from ethyl acetate in oily drops which slowly crystallize After washing with acetone the salt had mp $81-86^{\circ}$ (Found C, 645, H, 103 $C_{26}H_{56}N_{2,2}C_{1}H_{6}O_{4}$ requires C, 645, H, 108%)

SUMMARY

- 1 Several series of secondary diamines formally related to emetine have been prepared and tested against *Entamoeba histolytica* both *in vitro* and *in vivo*
- 2 Selected members of the series have also been tested against experimental infections with trypanosomes, leishmania and malaria
- 3 Bis (β 3 4 dimethoxyphenylethylamino) alkanes in which the hydrocarbon chain contained 6 to 10 carbon atoms were active. The corresponding 4-monomethoxy compounds were less active.
- 4 Bis (β phenylethylamino) alkanes also showed activity which was increased by the introduction of chlorine into the *ortho* or *para* positions of the benzene ring. The *ortho* compound was most active but also more toxic. Bis(phenylalkylamino)alkanes with either a greater number or fewer carbon atoms between the nucleus and the amino group were less effective.
- 5 Bis(alkylamino)alkanes containing 7 or 8 carbon atoms in the alkyl groups and 6 to 10 carbon atoms in the connecting chain also showed activity. This was slightly higher m vivo than that of the bis(β -phenylethylamino)alkane series but results at low dosc-levels were erratic

We are indebted to Dr H King for a gift of 1 10-bis(n-amylamino)decane dihydrochloride (No 65) Our thanks are also due to Messrs P Amsden, P Hankin, J M Judd, R Nicholson, R Penfound, F J Peters, K Pratley, A G Turner, C F Varney and the Misses S Pluthero and R Shipman for much assistance in the experimental work

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THE CHEMOTHERAPY OF AMOEBIASIS

PART III VARIANTS OF BIS(DIAMYLAMINO)DECANE

BY

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1 11-Diamidinoundecane (I) and 1 10-bis(din-amylamino)decane (II) are similarly constituted in that both contain two terminal basic radicals separated by a long chain of methylene groups. The former was shown by King, Lourie, and Yorke (1937) to be an active trypanocide in high dilution and the latter by Pyman (1937) to be an active amoebicide. Later Ashley, Barber, Ewins, Newbery, and Self (1942) modified the structure

of (I) by interrupting the chain with phenyl and phenylether groups, and obtained such compounds as 4 4'-diamidinostilbene ("stilbamidine") (III), 4 4'-diamidinodiphenoxy-propane ("propamidine") (IV) and -pentane ("pentamidine") (V) which were shown by Lourie and Yorke (1939) to have greatly enhanced trypanocidal activity

We have now studied the effect on amoebicidal activity of similar changes in the structure of 1 10-bis(di-n-amylamino)decane, and of the corresponding secondary bases. A few derived quaternary ammonium salts have also been included. The methods of testing were those described in Part I by Goodwin, Hoare, and Sharp (1948), and the results are given in Table I

DISCUSSION OF RESULTS IN TABLE I

The results with the standard substances 1 10-bis(di-n-butylamino)decane (No 64) and 1 10-bis (di-n-amylamino)decane (No 65, II) showed the compounds to be much less active in vitro than Pyman's results indicated. This may be due to the fact that the cultures used by Pyman contained a mixed bactericidal flora, whereas our tests were made upon a culture of amoebae with a single strain of Bact coli. These compounds had no significant activity in vivo

The tertiary aromatic amines (Nos 67–73) were found to be completely inactive, both *in vitro* at a concentration of 10⁻⁴ and *in vivo* in high doses, and a similar inactivity was found in the series of tertiary aromatic amines containing ether groupings (Nos 74–81) These are weak bases, but the more strongly basic tertiary araliphatic compounds (Nos 82–84) also showed no significant activity

Since all the active compounds recorded in Part II were secondary amines a number of secondary aromatic amines were prepared (Nos 85–92), here a slight *in vitro* activity became apparent when the alkyl group was amyl (No 92) or heptyl (No 89), but the compounds had practically no action *in vivo* Nos 86, 87, and 90, which, in addition to the two secondary amino-groups, carry two tertiary amino-groups, again had but slight activity. The length of the chain connecting the benzene rings seems to have little or no influence in these series

In the group of secondary araliphatic amines (Nos 93 to 106), the majority of the compounds had a moderate degree of *in vitro* activity, two showed a slight *in vivo* activity at high dose levels (Nos 103 and 104), and in two cases (Nos 100 and 105) there was a high *in vitro* activity but no action *in vivo* This group shows clearly that the *in vitro* test taken alone is inadequate as a means of assessing the value of a new drug. In the above instance activity is shown by a group of compounds therapeutically useless, in Part I it

TABLE I

Columns 6 and 7 "+" signifies improvement, "-" no Column 12 "1" signifies no activity against T equiperdum "2" ", ", " T rhodesiense "3" ", ", ", T congolense "4" ", ", ", T cruzi "5" ", ", ", " Leishimania donovani gallinaceum

8											
		App LD	50	Amoebicidal tests							.]
	Ref	(mg				n vivo	!	·	in vitro		- Other
Substance	No	Oral	Sub- cut	% diet	cond		Rats clear	%	Amoe- bicidal conc	Bact -cidal conc	tests
(1)	(2)	(3)	(4)	(5)	Walls (6)	Conts (7)	(8)	(9)	(10)	(11)	(12)
(C ₄ H ₉)_N(CH ₂) ₁₀ N(C ₄ H ₉) ₂ , 2C ₄ H ₆ O ₄	64	500	930	0 5	_		3/6	50	10-1	-	
$(C_5H_{11})_2N(CH_4)_{10}N(C_5H_{11})_2$, 2HCl	65			0 5 0 2 0 1	+ + -	++	3/7 7/14 3/12	40 50 25	10-5	_	
Tertiary aromatic amines											
$\left(\left(C_4 H_0 \right)_2 N \right)_2$, 2HCl	67	180	> 2000	0 5 0 2 0 1	 - -	<u>-</u>	0/3 0/4 0/7	0 0 0	-		1,3,4, 5,6
$\left((C_5H_{11})_2N\right)_2$, 2HCl	68	2000	2000	0 5	_	-	8/11	75	_	_	
$\left((C_4H_9)_2N\right)_2$ CH ₂ , 2HCl	69	950	> 2000	0 5 0 2 0 1	Toxic —		2/8 0/7	25 0	_	_	1,4,6
$\left((C_5H_{11})_2N\right)_2$ CH ₂ , 2HCl	70			0 5		_	6/13	45	_	_	5
$\left((C_1H_9)_2N\right)_2$ (CH ₂) ₂ , 2HCl	71	350	>2000	0 1	_		3/10	30		_	1,6
$\left(C_{5}H_{11}\right)_{2}N$ (CH ₂) ₂ , 2HCl	72	>2000	>2000	0 5	_		1/5	20		_	1,3
$\left((C_4 H_9)_2 N \right)_2 CH = \int_2 2HCI$	73	350	> 2000	02	_	_	0/6	0	_		1,5,6
Tertiary amino phenyl ethers											
$\left((C_4H_9)_2N \right)_2$ O, 2HCl	74	230	>2000	0 5 0 2		_	0/6 1/5	0 20	_		
$\left((C_{\delta}H_{11})_{2}N\right)_{2}O, 2HCI$	75	165	>2000	0 5	-		0/6	0	_		1,3
$\left((C_4H_{\theta})_2N\right)_2CH_2$, 2HCl	76	2000	>2000	0 5	-	_	0/4	0	-	_	1,3

TABLE 1-continued

		App	гох			Amoe	bicida!	l tests	<u> </u>		
	D.C	LÈ (mg)50 /kg)		17:	i vivo			in vitro		Other tests
Substance	Ref No	Oral	Sub- cut	% Cae		ıtıon	Rats clear	%	Amoe- bicidal conc	Bact. -cidal conc	
(1)	(2)	(3)	(4)	(5)	Walls (6)	Conts (7)	(8)	(9)	(10)	(11)	(12)
$\left((C_{\delta}H_{11})_{2}N \right)_{2}CH_{2}, 2HCI$	77	470	350	05 02	<u>-</u>	_	4/7 3/5	55 60	_		
$\left((C_1H_9)_2N\right)_2$ (CH ₂), 2HCl	78	600	>2000	0 2 0 1	 	<u></u>	2/8 0/8	25 0			4,5
$\left((C_bH_{11})_LNO-\right)_2(CH_2)_2$, 2HCl	79	800	>2000	0 5 0.2 0 1	- + -	- + -	1/3 0/4 3/7	35 0 40		_	1,3, 5,6
$\left((C_4H_0)_2N\right)_2(CH_2)_3$, 2HCl	80	2000	>2000	0 5	-		2/4	50	-	_	
$(C_5H_{11})_2N$ $(CH_2)_3$, 2HCl	81	950	> 2000	0 5	_	_	2/8	25	_		-
$\left((C_2H_5)_2N(CH_2)_2O\right)_2$, 2HCl	82	70	270	0 2			0/6	0	_	_	1,3,5
Tertiary araliphatic amines $\left((C_4H_9)_2N\ CH_2\right)_2, 2HBr$	83	350	>1000	0 5 0 2	Toxic		1/8	10			4,6
$\left(\begin{array}{c} \left(\begin{array}{c} \left(\left(\begin{array}{c} \left(\left(\begin{array}{c} \left(\left(\begin{array}{c} \left(\left(\left(\left(\left(\left(\left(\left(\left(\left(\left(\left(\left(\left(\left(\left(\left(\left(\left($	84			05	+	+	Toxic 3/3 0/8	100 0	10-4		
Secondary aromatic amines (C ₄ H ₉ NH , 2HCl	85	250	>2000	02	_		0/6	0	-	_	1,5,6
$\left((C_2H_8)_{\perp}N(CH)_{\perp}NH\left(\right) \right)_{2}$, 3HCl	86	570	330	0 5 0.2	_	_	1/5 1/5	20 20	10-4		2,3
$\left((C_2H_5)_2N(CH_2)_3NH \right)_2$, 4HBr	87			0502	+	+	3/8 2/7	40 30	10-4	_	(6)
(C ₄ H ₆ NH) CH ₂ , 2HCl	88	300	>2000	0.2	=	_	0/3 1/9	0 10			1,6
(C-H ₁₆ NH) CH_, 2HCl	_ 89	>2000	>2000	0 5 0.2 0 1	+ -	+	Tox 2/8 0/6	1c -25 0	_	_	

TABLE I-continued

	Approx			1	-						
	Ref	LD50 (mg/kg)				in vivo	in vi				
Substance	No	Oral	Sub- cut	% diet	cond	ecal lition	Rats clear	%	Amoe- bicidal conc	Bact -cidal conc	Other tests
(1)	(2)	(3)	(4)	(5)	Walls (6)	Conts (7)	(8)	(9)	(10)	(11)	(12)
$\left((C_2H_5)_2N(CH_2)_2NH\left(\right)\right)_2$ CH ₂ , 4HCl	90		1400	05	_		0/6	0	10-4	_	5,6
$\left(C_{1}H_{9}NH\right)_{2}$ (CH-) ₂ , 2HCl	91									-	6
$\left(C_{5}H_{11}NH\right)_{2}$ (CH ₂) ₂ , 2HCl	92	>2000	> 2000	0 5 0 2	+	+	1/4 4/5	25 80	10-4	<u> </u>	1,3,6
Secondary araliphatic amines											
C₃H₁NH CH₂ CH₂NH C₃H-, 2HCI	93	550	450	0 15	-	-	0/6	0	-		6
C ₄ H ₆ NH CH ₂ CH ₂ NH C ₄ H ₆ , 2HCl	94	350	310	0 5 0 2	_ _	_	2/6 1/5	35 20	_	-	2,3,6
C ₅ H ₁₁ NH CH ₂ CH ₂ NH C ₅ H ₁₁ , 2HCI	95	230	175	0 5	-	-	0/4	0		~	2,3
$\left(C_1H_9NH\ CH_2\right)_2$, 2HCl	96	1500	1500	0 2		_	1/3	35	10-4		2,3,6
(NH CH ₂), 2HCI	97	930	810	0 5 0 2 0 1	 	_ _	Toxic 2/5 3/7	40 40	10-4		······································
$\left(C_4H_9NH\ CH_2\right)_2$ CH ₂ , 2HCl	98			0 <i>5</i> 0 2			0/3 2/4	0 50	10-4	-	1,3
$\left(C_{b}H_{11}NHCH_{2}\right)_{2}CH_{2}$, 2HCl	99	933	574	0 2	_		0/4	0	10-4		6
NH CH ₂ CH ₂ , 2HCl	100			0 5	-	-	2/4	50	_		5,6
CH ₂ NH CH ₂ CH ₂ ,2HCl	101	>2000	>2000	0.5		-	0/4	0	10-4		
(C ₃ H ₇ NH CH ₃) ₂ , 2HCl	102	>2000	270	02	-	-	2/13	15			2,3
$\left(C_{4}H_{9}NH.CH_{2}\right)_{2}$ (CH ₂) ₂ , 2HCl	103	80	120	0 5 0 2	_	_	5/8 0/4	60	10-4	-	

TABLE I-continued

		App	rox	Amoebicidal tests									
Substance		LD50 (mg/kg)			i	n vivo	1	1	in vii	ro	Other		
		Oral	Sub- cut	% diet		ecal dition	Rats clear	%	Amoe- bicidal conc	Bact -cidal conc	tests		
(1)	(2)	(3)	(4)	(5)	Walls (6)	Conts (7)	(8)	(9)	(10)	(11)	(12)		
$\left(C_{5}H_{11}NHCH_{2}\right)_{2}$ (CH ₂) ₂ , 2HCl	104		250	0 5 0.2	+	+	3/4 1/8	75 10	10-5				
$\left(C_7H_{15}NHCH_2\right)_2$ (CH ₂) ₂ , 2HCl	105	>2000	>2000	02	_		0/1 T	oxic	10-5		6		
NH CH ₂ (CH ₂) ₂ , 2HCl	106	470	1000	0 5 0 2 0 1	Toxic + +	+ +	2/5 4/7	40 55	10 ⁸		_		
Quaternary ammonum salts $\begin{pmatrix} C_4H_9 & + \\ C_4H_9 & + \\ C_4H_9 & + \end{pmatrix} I^-$ $C_4H_9 & + \\ C_4H_9 & +$	107	>2000	550	0 5 0 2	Toxic		4/5	80	10 4		3		
$\begin{pmatrix} C_{5}H_{11} & + \\ C_{5}H_{11} - N & - \\ CH_{3} & - \end{pmatrix}_{2} CH_{2} C\overline{l_{2}}$	108	440	100	0 2 0 1 0 05	+ -	-	6/6 0/4 0/2	100 0 0	10-4		2,3		
" (CH ₂)_ Ī ₂	109	20		05	_	-	1/10	10	10-4		1,3,6		
CH ₃ (+ N-CH ₂ CH ₂ Cl ₂	110			0 5	_	- ,	4/8	50					

was shown that diodoquin, although active in vivo had no appreciable in vitro activity

The quarternary ammonium salts (Nos 107–110) were slightly more active than the corresponding tertiary bases (Nos 67, 70, 72, and 84)

CHEMICAL SECTION

Aromatic amines

NNN'N'-Tetra-n-buty Ibenzidine (No 67) Benzidine (184 g, anhydrous) was refluxed in n-butanol with a 33% excess of n-butylbromide (7 3 g) and potassium carbonate (3 7 g) for 16 hours. The filtered solution was evaporated and the residue shaken with ether and aqueous sodium hydroxide to isolate the crude product which was then dissolved in light petroleum (bp 40-60°) and passed through a column of activated alumina. The base (3 2 g, 78%), which exhibited a blue fluorescence in organic solvents, was obtained on evaporation of the petrol solution. It crystallized from alcohol in flakes mp 58 5° (Found C, 82 2 H, 10 8 C₇₈H₄₁N₂.

requires C, 82 3, H, 10 9%) The dipicrate, which crystallized in yellow flakes from alcohol, had mp 203–204° (decomp) (Found C, 55 4, H, 5 9, N, 12 9 C₂₈H₄₄N₂, 2C₆H₃O₇N₃ requires C, 55 4, H, 5 8, N, 12.9%) The dihydrochloride, mp 240–250° (decomp), was readily soluble in alcohol but suffered partial hydrolysis in aqueous solution (Found N, 6 1, Cl, 14 9 C₂₈H₄₁N₋, 2HCl requires N, 5 8, Cl, 14 8%) The base reacted with methyl iodide in benzene to form a monomethiodide (No 107) which crystallized from ethanol, mp 146–147° (after drying at 100° in vacuo) (Found C, 63 3, H, 8 5, I, 24 3 C₄₈H₄₄N₂, CH₃I requires C, 63 3, H, 8 6 I, 23 1%)

An alternative method of obtaining tetra-n butylbenzidine utilized the oxidative procedure of Ullmann and Dieterle (1904) and of Frohlich (1911) Di-n-butylamline (4 1 g), concentrated sulphuric acid (20 g), and turpentine (0 1 g) were heated together at 190–200° for 4½ hours. The mixture was made alkaline and, after removal of unchanged dibutylaniline by steam-distillation, the residual mass was dried, powdered and extracted with

benzene (Soxhlet) The dark extract was evaporated and the residue, decolorized by filtration of its solution in light petroleum through alumina, consisted of NNN'N'-tetra-n-butylbenzidine (2 6 g, 64%)

NN'-Di-n-butylbenzidire (No 85) When benzidine was butylated with only half the above quantity of *n*-butyl bromide the resulting mixture of bases was only partially soluble in light petroleum (b p 40-60°) When the undissolved substance was dissolved in benzene and passed through a column of alumina some unchanged benzidine remained in the column, while the benzene carried through NN'-di-n-butylbenzidine, which crystallized from alcohol in laminae mp 72° (Found C, 810, H, 98 C₀H₂₈N₂ requires C, 810, H, 95%) The dihydrochloride crystallized from ethanol in fine needles mp ca 280° (decomp) (Found C 644, H, 84 C₀H₂₈N₂,2HCl requires C, 650, H, 82%)

The base was also obtained in small yield by the oxidation of *n*-butylaniline with sulphuric acid in the manner previously described

NNN'N'-Tetra-n-amylbenzidine (No 68) The amylation of benzidine was carried out in a similar manner to the butylation, using a 33% excess of n-amylbromide in n-butanol. The base obtained on removal of the petroleum was distilled at ca 270°/0.5 mm, and was obtained as an oil. (Found. C, 83.0, H, 11.5, N, 6.3. $C_{\pi_c}H_{52}N_2$ requires C, 82.7, H, 11.3, N, 6.0%). The dipicrate crystallized from ethanol in small yellow prisms mp. 186–187°. (Found. C, 57.4, H, 6.3, N, 12.5. $C_{3^{\circ}}H_{12}N_{\circ}$, 2 $C_{6}H_{3}O_{7}N_{3}$ requires C, 57.3, H, 6.3, N, 12.1%). The dihi drochloride, mp. 230–235°. (decomp.), crystallized from ethanol on addition of ether. (Found. C, 70.9, H, 10.0. $C_{32}H_{52}N_{2}$, 2HCl requires C, 71.5, H, 10.1%).

Bis(p NN-di-n-butvlaminophenyl)methane (No 69) This was prepared according to Reid and Lynch (1936) and, in 75% yield, by the butylation of bis(p-aminophenyl)methane The base was obtained as an oil, b p 280–320°/20 mm (Found C, 82 7, H, 11 4 Calc C, 82 4, H, 11 0%) The dipicrate, golden flakes from ethanol, had m p 183–184° (Reid and Lynch, 1936, recorded m p 156°) (Found C, 55 8, H, 5 9, N, 12 8 Calc C, 55 9, H, 60, N, 12 7%) The dihydrochloride m p ca 220° (decomp) was very soluble in ethanol and in water (Found Cl, 14 6 C₂₉H₁₆N₂,2HCl requires Cl, 14 3%)

Bis(p-NN-di-n-amylaminophenyl)methane (No 70) The base, obtained by amylation of bis(p-aminophenyl)methane with a 33% excess of n-amylbromide in the manner previously described, formed a viscous oil (Found C, 83 1, H, 11 2 $C_{33}H_{54}N_2$ requires C, 82 8, H, 11 4%) The dihvdro-hloride crystallized from ethanol, mp 203° (decomp) (Found Cl, 13 0 $C_{33}H_{54}N_2$,2HCl requires Cl, 12 9%) The dimethodide, flat jagged needles from ethanol, had mp ca 140° in a sealed tube (softening at 70-75°) (Found N, 3 8, I, 3 3 4 $C_{33}H_{54}N_2$,2CH₃I requires N, 3 7, I, 33 3%) The dimethochloride (No 108) was prepared from this by the usual procedure

Butylation of α β -bis(p-aminophenyl)ethane Butylation carried out by the standard method previously described gave an oil which distilled at ca 230°/0 01 mm

The distillate, α β -bis(p-NN-di-n-bitylammophenyl)ethane (No 71), crystallized from ethanol in laminae m p 3 4 5–35 5° (Found C, 82 5, H, 11 1, N, 6 7 $C_{30}H_{48}N_2$ requires C, 82 5, H, 11 1, N, 6 4%) The base and its solutions in ether or alcohol exhibited a strong violet fluorescence The dipicrate crystallized from ethanol in golden needles or laminae m p 173 5–174 5° (slow heating) with rapid heating dimorphic change occurred at 174–175° and the m p was 188–189° (Found C, 56 7, H, 6 3, N, 12 6 $C_{30}H_{48}N_2$, 2C₆H₃O-N₃ requires C, 56 4, H, 6 1 N, 12 5%) The dihydrochloride, soluble in ethanol and in water, had m p ca 231° (decomp) (Found Cl, 13 9 $C_{30}H_{48}N_2$, 2HCl requires Cl, 13 1%)

When α β -bis(p-aminophenyl)ethane (11.5 g) was heated with *n*-butylbromide (35 g, 16% excess) in an autoclave at 200° for 40 hours the resulting mixture of bases reacted readily with acetic anhydride to give an oily product which was treated several times with warm light petroleum (b p 40-60°) Solid material crystallized from the petrol on standing and, on evaporation, the filtered solution gave an oil which was mainly \alpha \betabis(p-NN-di-n-butylaminophenyl)ethane The material (12 8 g) was crystallized from benzene/light petroleum to give α β-bis(p-N-n-butylacetamidophenyl)ethane in needles m p 85-86° (Found C, 762, H, 90; N, 68 C₂₆H₃₆O₂N₂ requires C, 764, H, 89, N, 69%) On deacetylation with alcoholic hydrochloric acid this derivative gave α β-bis(p-N-n-butylaminopheryl)ethane (No 91) which formed crystals m p 86-87° from aqueous ethanol (Found C, 813, H, 96, N, 86 requires C, 81 5, H, 99, N, 86%) The dihydrochloride had mp ca 235° (decomp) (Found Cl. 183. C22H32N2,2HCl requires Cl, 178%)

Amylation of α β -bis(p-aminophenyl)ethane standard amylation procedure did not in this case bring about complete reaction, a further treatment with n-amyl bromide was found to be necessary σ β-Bis(p-NN-di-n-amylaminophenvl)ethane (No 72) crystallized from 80% alcohol in jagged needles mp 40° showing a strong violet fluorescence (Found C, 830, H, 116. $C_{34}H_{56}N_2$ requires C, 830, H, 115%) The divicrate, yellow needles from ethanol, had mp 207-208° (Found C, 580, H, 67, N, 121 $C_{34}H_{56}N_2, 2C_6H_3O_7N_3$ requires C, 581, H, 66, N, 118%), and was used in separating the pure base from the incompletely amylated The dihydrochloride had mp 214reaction product (Found Cl, 124 C₃₄H₅₆N₂,2HCl requires Cl, 125%) The dimethiodide (No 109) separated as a crystalline powder m p 158-160° (decomp) on addition of benzene to its alcoholic solution (Found C, 552; $C_{34}H_{56}^4N_2$, 2CH₃I requires C, 557, H, 80, I, 323 H, 81 I, 327%)

When the products of the incomplete reaction in light petroleum (b p 40–60°) were passed through a column of alumina, α β -bis(p-N-n-amvlaminonhenyl)ethane (No 92) was adsorbed. The base was recovered by elution with ether/light petroleum and crystallized from ethanol in laminae mp 86° (Found C, 81 8, H, 10 2, N, 8 2 $C_{21}H_{36}N_2$ requires C, 81 8, H, 10 3, N, 80%) The dihydrochloride had mp ca 240° (decomp) (Found:

N, 70, CI, 170 $C_{24}H_{36}N_2$,2HCl requires N, 66, Cl. 167%)

Trans-4 4'-bis(di-n-butylamino)stilbene (No 73) This was obtained on applying the standard butvlation procedure to trans-4 4'-diaminostilbene The base. which exhibited a strong violet fluorescence in organic solvents, crystallized from ethanol in opaque laminae mp 70-71° (Found C, 828, H, 107, N, 66 C₃₀H₄₈N₂ requires C, 829, H, 107, N, 65%) The dinicrate, small golden needles from much ethanol, had m p ca 213° (decomp) (Found C, 566, H 60, N 12 4 C₃₀H₄₆N ,2C₆H₃O₇N₃ requires C, 56 5, H, 5 9. N, 126%) The dihydrochloride had mp 240-242° (Found N, 57 Cl, 139 C₁₀H₁₆N₀,2HCl requires N. 55, Cl. 140%)

Tertiary aromatic amines derived from phenyl ethers

Bis(p-amanophenoxy)alkanes The bis(p-aminophenoxy)alkanes used were prepared by the method described by Kinzel (1898) for the preparation of α B bis(pay-Bis(p-nitrophenoxy)propane aminophenoxy)ethane from sodium p-nitrophenate and 1 3 dibromopropane forms anhydrous crystals from alcohol, mp 132° (Found C, 566, H, 48, N 88 C₁₅H₁₄O₆N₅ requires C, 56 6, H, 4 4, N, 8 8%) αγ-Bis(p-acetamidophenyl)propane, mp 183°, was obtained in 87% yield by reduction of the above nitro compound with powdered iron and acetic acid (99%) On hydrolysis with hydrochloric acid it yielded ay-bis(p-aminophenyl)propane dihvdrochloride, mp 268° after recrystallization from water, yield 75% (Found C, 546, H, 62, N, 82, Cl. 21 4 C₁₅H₁₈O N₂,2HCl requires C, 54 4, H, 61, N, 85, Cl, 214%)

Bis(dialk vlaminophenyl)ethers (Nos 74-81) The general method for the preparation of these compounds is illustrated by the following example. To a boiling solution of 4 4'-diaminodiphenylether (4 g) and n-butylbromide (22 g) in alcohol (25 cc), a solution of

sodium (2 5 g) in alcohol (80 cc) was added at such a rate as to maintain a slightly alkaline reaction. After 12 hours the alcohol was removed and the residue diluted with water and extracted with ether. After distilling off the solvent the base was dissolved in light petroleum (b p 40-60°), passed through a column of alumina and purified as picrate. The picrate was then converted to hydrochloride in the usual manner. The properties of the compounds are given in Table II

4 4'-Bis(B-diethylaminoethoxy)diphenyl (No 82) 4 4'-Dihydroxydiphenyl (6.2 g) was treated with sodium (1 6 g) in ethanolic solution. The solution was refluxed overnight with β-diethylaminoethyl chloride (from 143 g of the hydrochloride) The concentrated solution was made alkaline and extracted with ether, the resulting oil (3.5 g.) dissolved in light petroleum (b.p. 40-60°) was passed through a column of alumina The base (2.9 g. 23%) obtained on removal of the solvent gave crystals. mp 45 5°, from aqueous alcohol (Found C. 750, H. 94 N. 74 - Co. H. 200 No requires C. 750, H. 94, The dihydrochloride had mp 244-245° N. 73%) (Found C, 634, H, 86, Cl, 157 (decomp) C₂H₃₈O₂N₂.2HCl requires C. 63 0, H. 8 4 Cl. 15 5%)

Tertiary araliphatic amines

4 4'-Bis(di-n-butylaminomethyl)diphenvl (No 83) 4 4'-Bis(bromomethyl)diphenyl (3 4 g) and dibutylamine (2 75 g) in toluene (50 cc.) were heated under reflux for 10 hours. The somewhat only solid was filtered off, washed with hot toluene and recrystallized from acetone. The dihydrobromide separated in long shining rods, mp 227° (Found C, 60 45, H, 9 0,- Br, 26.3 C₃₀H₄₈N₂,2HBr requires C, 60 2, H, 8 4 Br, 26 7%)

4 4'-Bis(N-piperidinomethyl)diphenylmethane (No 84) A solution of 4 4'-bis(bromomethyl)diphenylmethane (71 g.) and piperidine (3 4 g) in ethanol (35 cc) was refluxed for 7 hours, concentrated, and shaken with 10% sodium hydroxide and benzene The benzene solution

TABLE II

Compound				Fou	nd %		Famoula	Requires %			
Compound	No	mp	С	H	N	Cl	Formula-	C	Н	N	
Bis(p-di-n-but) laminophenyl) ether, 2HCl do dipicrate Bis(p-di-n-amylaminophenyl) ether dipicrate Bis(p-di-n-but) laminophenox) methane 2HCl do dipicrate Bis(p di-n-amylaminopheroxy) methane 2HCl do dipicrate αβ-Bis(p-di-n-butylaminophenoxy) ethane 2HCl do dipicrate αβ-Bis(p-di-n-amylaminophenoxy) ethane 2HCl do dipicrate αγ-Bis(p-di-n-butylaminophenoxy) ethane 2HCl do dipicrate αγ-Bis(p-di-n-butylaminophenoxy) propane 2HCl do dipicrate αγ-Bis(p-di-n-amylaminophenoxy) propane 2HCl do dipicrate	74 75 76 77 78 79 80 81	182° 148° 88°1 168° 84°2 140° 205°3 156° 181° 170° 169°4 202° 94° 198°	65 5 54 4 56 2 62 3 53 95 63 95 55 5 54 4 68 0 56 3 66 8 54 9 56 95	93 57 62 94 565 95 62 59 67 93 59	13 3 12 3 5 15 12 4 4 7 12 1 5.2 11 9 5 0 11 6 5 0 12 3 4 8 11.25	13 9 12 7 11 5 13 1 11 4 12 6 11 5	C ₂₈ H ₄₄ ON ₂ ,2HCl, H ₂ O C ₂₈ H ₄₄ ON ₂ ,2C ₄ H ₃ O ₇ N ₃ C ₃₂ H ₅₂ ON ₂ ,2C ₄ H ₃ O ₇ N ₃ C ₂₉ H ₄₆ O ₂ N ₂ ,2HCl,2H ₂ O C ₂₉ H ₄₆ O ₂ N ₂ ,2C ₅ H ₃ O ₇ N ₃ C ₃₃ H ₅₄ O ₃ N ₇ ,2HCl,2H ₂ O C ₃₃ H ₅₄ O ₂ N ₂ ,2C ₅ H ₃ O ₇ N ₃ C ₃₀ H ₆₀ O ₂ N ₂ ,2C ₅ H ₃ O ₇ N ₃ C ₃₄ H ₅₀ O ₂ N ₂ ,2C ₅ H ₃ O ₇ N ₃ C ₃₄ H ₅₀ O ₂ N ₂ ,2C ₅ H ₃ O ₇ N ₃ C ₃₄ H ₅₀ O ₂ N ₂ ,2C ₅ H ₃ O ₇ N ₃ C ₃₁ H ₅₀ O ₂ N ₂ ,2C ₅ H ₃ O ₇ N ₃ C ₃₁ H ₅₀ O ₃ N ₂ ,2C ₅ H ₃ O ₇ N ₃ C ₃₁ H ₅₀ O ₃ N ₂ ,2C ₅ H ₃ O ₇ N ₃ C ₃₅ H ₅₅ O ₂ N ₂ ,2C ₅ H ₃ O ₇ N ₃	65 2 54 4 56 3 62.0 53.95 63 95 55 9 54 4 68 3 56 2 67 0 54 9	94 57 6.2 90 57 98 6.2 59 98 64 94 60 65	12.7 11.5 50 12.3 4.5 11.6 5.2 12.1 4.7 11.6 5.0 11.9 4.6 11.2	

^{&#}x27;The anhydrous salt melts at 130°

gave an oil (3 1 g) which was dissolved in light petroleum (b p 40-60°) and filtered through alumina. The resulting base, after distillation at 230°/0 5 mm, formed crystals mp 63-65° from aqueous ethanol. (Found. C, 82 0, H, 8 9, N, 7 5. C₂₅H₃₄N₂ requires C, 82 8, H, 9 5, N, 7 7%). The dipicrate, orange-yellow needles from much ethanol, had mp 181-182° (after drying at 100° in vacuo). (Found. N, 13 8. C₂₅H₃₄N₂,2C₆H₃O-N₃ requires N, 13 7%). The dihydrochloride crystallized from alcohol-ether, mp ca 300° (decomp.). (Found. C, 68 8, H, 7 8, Cl, 16 0. C₂₅H₃₁N₂,2HCl requires C, 68 9, H, 8 3, Cl, 16 3%). The dimethodide separated from alcohol-benzene in yellowish crystals, mp. 175° (decomp.). (Found. C, 50 6, H, 6.2. C₂₅H₃₁N₂,2CH₃I requires C, 50 2, H, 6.2%).

Secondary aromatic amines

NN'-Bis(β-dieth) laminoethy l)benzidine (No 86) Benzidine (4 4 g) was refluxed overnight with β-diethylaminoethyl chloride (from 12 g of the hydrochloride) and potassium carbonate (5 g) in xylene The solvent was removed by steam-distillation and a basic oil (11 g) recovered by extraction with ether from alkaline solution Partial purification was effected by passing the oil dissolved in light petroleum (b p 40-60°) through a column of alumina The product (9 4 g) was treated in ethanol with picric acid (11.5 g) and the oily picrate which separated was extracted several times with acetone/ethanol The extracts, on evaporation and basification, gave an oil from which an ether-insoluble hydrochloride (3 8 g) was obtained The base was obtained from this by treatment with ammonia, it crystallized in soft flakes m p 70° from aqueous ethanol (Found C, 753, H, 97, N, 146 C₂₄H₂₈N₄ requires C, 753, H, 100, N, 147%) The truhydrochloride, soluble in ethanol, had mp ca 235° (Found N, 218, Cl, 11 5 C₂₄H₃₈N₄,3HCl requires N, 21 6, Cl, 11 4%)

NN'-Bis(γ-diethvlaminopropyl)benzidine (No 87) Benzidine (45 g) was refluxed with γ-diethylaminopropyl chloride and potassium carbonate in n-butanol, the method of procedure being that of the previous experiment. The oil (60 g) obtained from the petrol solution gave a picrate (34 g) when treated with picric acid (43 g) in ethanol. The base (15 g) obtained from the picrate was distilled at ca 320°/04 mm. (Found C, 764, H, 114, N, 133 C₂₆H₄₂N₄ requires C, 761, H, 103, N, 136%) It formed a tetrahydrobromide as reported by Work (1940) and a tripicrate which separated as golden-yellow crystals mp 125° (decomp.) from acetone (Found C, 464, H, 48, N, 158 C₂₆H₄₂N₄, 3C₆H₃O₇N₃,2H₂O requires C, 466, H, 47, N, 161%)

Bis(p-N-n-butylaminophenyl)methane (No 88) This compound was obtained from n-butylaniline and formaldehyde as a crystalline solid mp 44–45° (Wagner, 1934, gave mp 41–42°, Reid and Lynch, 1936, recorded mp 45°) The dihydrochloride had mp 160–162° (Found C, 65 4, H, 8 5 C₂₁H₃₀N₂,2HCl requires C, 65 7, H, 8 4%)

Bis(p-N-n-heptvlaminophenyl) methane (No 89) Bis(p-m) aminophenyl) methane (2 47 g) was boiled with sodamide (1 g.) in dry xylene for 4 hours, n-heptylbromide (4 5 g)

was added, and the mixture refluxed overnight solvent was removed by steam-distillation and the reaction product extracted with ether from alkaline solution The resulting dark-coloured oil was dissolved in light petroleum (b p 40-60°) and the solution passed twice through a column of alumina The colourless oil (40 g) obtained on removal of solvent was distilled at 0 2 mm, collecting material distilling up to 280° This crude base was treated in ethanol with a little 10% hydrochloric acid, causing the separation of an oily hydrochloride which on standing at 0° formed a semi-This was pressed on a porous tile and solid mass crystallized from ethanol/ether to give bis(p-N-nheptylanur ophenyl)methane dihvdrochloride (0 9 g, 15%) m p 195-196° (Found C, 69 1, H, 9 5 $C_{27}H_{12}N_2$, 2HCl requires C, 69 4, H, 9 5%), which readily became blue in moist air The base was purified by alumina (in light petroleum/benzene) and formed crystals m p 48° from aqueous ethanol (Found C, 817, H, 107, N, 69 $C_{27}H_{42}N_2$ requires C, 822, H, 107, N, 71%)

Bis(p-β-diethylaminoethylaminophenyl)methane (No 90) Bis(p-aminophenyl)methane (9 9 g) was refluxed overnight with β-diethylaminoethyl chloride (from 18 1 g of the hydrochloride) and potassium carbonate (7 2 g) in ethanol. The resulting basic oil (17 g), purified by alumina (light petroleum solution) was distilled at 0 2 mm, collecting material (7 3 g) distilling above 240°. This product was purified by recrystallization of the tetrahydrochloride, mp ca 167–169° from ethanol. The salt was too deliquescent for satisfactory analyses to be obtained. The base was an oil b p ca 280°/0 2 mm (Found C, 75 8, H, 10 2, N, 13 7 $C_{25}H_{40}N_4$ requires C, 75 7, H, 10 2, N, 14 1%)

Secondary araliphatic amines

Preparation of aromatic bromomethyl compounds A modification of the chloromethylation procedure of Cambron (1939) was adopted for the bromomethylation of benzyl bromide, diphenyl, diphenylmethane and α β -diphenylethane The general method is illustrated by the following example Diphenylmethane (39 g) was added to a mixture of paraformaldehyde (30 g), hydrobromic acid (77 cc., d 1 7), phosphoric acid (57 cc., d 1 75) and glacial acetic acid (95 g) The solution was maintained at 95-110° for 5 hours while dry hydrogen bromide (20-30 g) was passed in, left overnight and heated for a further 8 hours The mixture was poured into water (1,000 c.c), and the pasty precipitate filtered off and crystallized from benzene 4 4'-Bisbromomethyldiphenylmethane (33 g, 40%) separated from benzene in long prisms m p 153 5° (Found C, 509, H, 40, Br, 451 C₁₅H₁₄Br₂ requires C, 51 0, H, 40, Br, 45 1%)

In a similar manner were prepared p-xylylene dibromide (34% yield), 4 4'-bisbromomethyldiphenyl (35%) (cf v Braun, 1937) and 4 4'-bisbromomethyl- α β -diphenylethane (34%) which crystallized from acetone in laminae in p 117-120° (Found C, 521, H, 45, Br, 431 $C_{16}H_{16}Br_2$ requires C, 522, H, 44, Br, 434%)

Preparation of aromatic alkylaminomethyl derivatives (Nos 93-106) Condensation of the bromomethyl compounds with alkylamines proceeded readily in such

solvents as alcohol, benzene or xylene, with or without the addition of sodium iodide. In all cases, however, the required product was accompanied by a considerable amount of amorphous substance of high molecular weight, apparently formed by condensation of the -NH groups in the product with further molecules of bromomethyl compound (cf v Braun, 1937), these rendered the purification of the product somewhat difficult The formation of compounds of high molecular weight was reduced by refluxing the bromomethyl compound with an excess (10 mols) of the alkylamine for 3-5 hours without other solvents. The excess of alkylamine was removed at 100° under reduced pressure and the residue shaken with 10% sodium hydroxide and ether The oil obtained on evaporation of the ether was then treated with light petroleum (b p 40-60°) in which all the bases.

with the exception of the 4 4'-bis(cyclohexylaminomethyl) derivatives of diphenyl and α β-diphenylethane, were very soluble, but in which the amorphous material was almost insoluble The petroleum solution was clarified by filtration through alumina, and evaporated resulting base was dissolved in ethanol and the solution, filtered through kieselguhr to remove further traces of amorphous material, was treated with hydrochloric acid (d 1 2) to precipitate the dihydrochloride This procedure was used in the preparation of all the compounds in Table III with the exception of two prepared in presence of a solvent The bases were redistilled or, in some cases. crystallized from aqueous ethanol from which they separated in laminae The dihydrochlorides were insoluble in ethanol but dissolved fairly readily in warm water to a neutral solution

TABLE III

Compound	No	bp ormp		Fou	nd %		Formula	-	Requ	ıres %		
Compound	110	op obm p	С	H	N	Cl	Politicia	С	Н	N	Cl	ĺ
l 4-Bis(n-propylaminomethyl)benzene do dihydrochloride l 4-Bis(n butylaminomethyl)benzene do dihydrochloride	94	b p ca 310° subl > 320° Oıl subl > 320°	76 5 57 8	10 9 8 9 9 1	8 4	24 2	C ₁₄ H ₂₄ N ₂ C ₁₄ H ₂₄ N ₂ ,2HCl C ₁₆ H ₂₈ N ₂ C ₁₆ H ₂₈ N ₂ ,2HCl	76 3 57 3	11 0 8 9 9 4	87	24 2	
1 4-Bis(n-amylaminomethyl)benzene do dihydrochloride 4 4'-Bis(n-butylaminomethyl)diphenyl do dihydrochloride	95 96	b p 150°/0 2mm decomp > 320° m p 43 5-45° decomp > 360°	78 2 62 3 81 6 66 6	11 6 10 0 10 0 8 5	10 1	20 4 17 7	C ₁₈ H ₃₂ N ₂ C ₁₈ H ₃₂ N ₂ ,2HCl C ₂₁ H ₃₂ N ₂	78 2 61 9 81 4 66 5	11 7 9 8 9 9 8 6	10 1	20 3 17 8	
4 4'-Bis(cyclohexylaminomethyl)dıphenyl do dihydrochloride 4 4'-Bıs(n-butvlaminomethyl)dıphenyl-		m p 94 5-95 5° decomp > 360°	83 8 69 3	98 88		15 6	C ₂₂ H ₃₂ N ₂ ,2HCl C ₂₆ H ₃₆ N ₂ C ₂₆ H ₃₆ N ₂ ,2HCl	82 9 69 5	96 85	0.2	15 8	
methane do dihydrochloride 4 4'-Bis(n-am) laminomethyl)diphenyl-		m p 9-10° decomp > 320°	80 9 67 4	10 4 8 8	8 2 6 6	170	C ₂₃ H ₃₄ N ₂ ,2HCl	81 6 67 2	10 1 8 8	83 68	17 2	
methane do dihydrochloride 4 4'-Bis(cyclohexylaminomethyl)diphenyl-		b p 238°/1 0mm decomp <i>ca</i> 327°	81 4 68 8	10 6 9 1	76		C ₂₅ H ₃₈ N ₂ C ₂₅ H ₃₈ N ₂ ,2HCl	81 9 68 3	10 5 9 2	76	~	
methane do dihydrochloride 4 4'-Bis(benzylaminomethyl)diphenylmethane do dihydrochloride	101	m p 37-38 5° decomp > 320° Oil decomp > 350°	83 8 70 2 85 3 72 0	98 89 77 67	71	15 1 14 4	C ₂₇ H ₂₈ N ₂ C ₂₇ H ₃₈ N ₂ ,2HCl C ₂₉ H ₃₀ N ₂ C ₂₉ H ₃₀ N ₂ ,2HCl	83 0 70 0 85 7 72 6	98 87 74 67	69	15 3 14 8	}
4 4'-Bis(n-propylaminomethyl)-α β- diphenylethane do dihydrochloride		m p 53-54 5° m p ca 345°	81 8 66 5	10 1 8 4		18 0	C ₂₂ H ₃₃ N ₂ C ₂₂ H ₃₃ N ₂ ,2HCl	81 4 66 5	9 9 8 6		178	5
4 4'Bis(n-butylammomethyl)-α β- diphenylethane do dihydrochloride 4 4'-Bis(n-amylammomethyl)-α β-		m p 46-47° decomp > 320°	81 8 67 9	10 4 9 1	79	16 7	C24H36N2 C24H36N4,2HCI	81 8 67 8	10 3 9 0	80	167	:
do dihy drochloride 4 4'-Bis(n-hepty laminomethyl)-α β-	104	m p 7–8° decomp 333°	82 3 68 9	10 6 9 0	74	15 6	C ₂₆ H ₄₀ N ₂ C ₂₆ H ₄₀ N ₂ ,2HCl	82 1 68 9	10 6 9 3	74	15 6	
diphenylethane do dihydrochloride 4 4'-Bis(cyclohex) lanunomethyl)-α β-	105	m p 35–36° decomp 341°	81 3 70 7	11 ₁ 3 10 5		13 9	C ₃₀ H ₄₈ N ₂ C ₂₀ H ₄₈ N ₂ ,2HCl	82 5 70 7	11 1 9 9		13 9	3
diphenylethane do diliydrochloride		m p 115-116° decomp > 360°	83 1 69 7	10 2 9 4		14 7	C ₂₈ H ₄₀ N ₂ C ₂₈ H ₄₀ N ₂ ,2HCl	83 1 70 4	10 0 8 9		148	El

^{*}By refluxing 4 4'-bischloromethyldiphenylmethane (7 7 g) with n-butylamine (7 0 g) and sodium bromide in xylene †By refluxing 4 4'-bisbromomethyldiphenylmethane (9 7 g) with benzylamine (7 0 g) and sodium iodide in xylene

SUMMARY

- I A series of tertiary aromatic diamines in which two aromatic rings are connected by alkylene chains or chains interrupted by ether groupings have been shown to have no significant amoebicidal activity either in vitro or in vivo
- 2 Some of the similarly constituted secondary diamines showed a slight activity in vitro
- 3 Most of the secondary araliphatic diamines tested showed some activity in vitro, but no activity in vivo except at toxic dose-levels
- 4 Quaternary ammonium salts were slightly more active than the corresponding tertiary bases

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THE TREATMENT OF "SHOCK" WITH SODIUM SALT SOLUTIONS

ВY

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Rosenthal (1942-5) This stated that the administration of large quantities of sodium salt solutions reduces the mortality in mice suffering from shock caused by thermal burns, trauma, and haemorrhage Fox (quoted by Rosenthal, 1943) in man, Allen (1943) using dogs, and Prinzmetal et al (1943) in mice, have also teported good results from the use of sodium chloride solutions for the treatment of shock due to thermal burns, while Cullumbine and Box (1946) have found that large quantities of sodium chloride or sodium lactate solution reduce the mortality from lewisite shock, but that different species are not equally responsive. These results have now been confirmed in mice suffering from shock caused by thermal burning and extended to rats, rabbits, and goats burnt with liquid mustard gas

THERMAL BURNING

Mice, weighing 18-20 g and under nembutal anaesthesia, were used and their hind limbs immersed for 30 sec in water which was maintained at a temperature of 80° C. Therapies were given at various time intervals after the immersion. The time of death after immersion for each mouse was carefully noted. Death occurred rapidly Q-4 hours) in 100 per cent of

untreated mice and an effort was made to find therapies which would significantly prolong the median survival time. The results were analysed by means of Fisher's method of analysis of variance for Latin squares

Therapy with sodium chloride—Table I details the results and conclusions of a typical experiment. Further experiments showed that therapies administered 30 to 60 min after burning produced better survival times than therapies administered immediately or 120 min after burning. In general, 0.45 per cent (w/v) NaCl solution was better than a 0.9 per cent solution which, in turn, was better than a 1.8 per cent solution All these salt solutions were better than water 'Similar results were obtained whether the sodium chloride solution was given by mouth or by intraperitoneal injection.

From the results of the above experiments, it was concluded that, of the therapies tried, 1 ml doses of the sodium chloride solutions administered 30 min after burning gave the best chance of survival. Therefore these therapies were assessed in less severely burned mice and the percentage mortalities of the treated and untreated groups compared.

The hind limbs of mice, under nembutal anaesthesia, were immersed for 30 sec in water maintained at 70° C Sodium chloride solutions were administered

TABLE I

EFFECT OF INTRAPERITONEAL INJECTION OF DIFFERENT CONCENTRATIONS OF SODIUM CHLORIDE IN AQUEOUS SOLUTION ON THE SURVIVAL TIME OF MICE SUFFERING FROM THERMAL BURNS

Therapy given 30 min after burning	Median survival time in hours (5 mice per group)	Conclusions
1 ml water 2 ml , 1 ml 1 8% NaCl 2 ml 1 8% NaCl 2 ml 1 8% NaCl 1 ml 0 9% NaCl 2 ml 0 9% NaCl 1 ml 0 45% NaCl 2 ml 0 45% NaCl 2 ml 0 45% NaCl Nil	10 0 3 2 9 9 3 8 13 5 9 5 20 0 15 2 2 5	 (1) In general 1 ml dosages are better than 2 ml dosages in prolonging survival time (P = 0 05) (2) 0 45% NaCl is better than 0 9% NaCl which in turn is better than 1 8% NaCl or water (3) All therapies but 2 ml water and 2 ml 1 8% NaCl produced a significant (P = 0 05) increase in median survival time

intraperitoneally 30 min later and the resultant total mortalities noted. These mortalities were as follows

Untreated mice - 24/25 1 ml of 18 per cent NaCl - 11/25 1 ml of 0.9 per cent NaCl - 9/25 1 ml of 0.45 per cent NaCl - 6/25

Hence all these therapies produced a significant reduction of the mortality, and the results agree substantially with those of Rosenthal

Therapy with carbohy drate and protein—It has been noted that, after thermal burns the carbohydrate stores (liver and muscle) quickly become exhausted (Clark and Rossiter, 1944) and a large negative nitrogen balance soon occurs (Cuthbertson et al. 1939 Clark, Peters and Rossiter, 1945, Taylor et al. 1943) Therefore the effectiveness of the early administration of aqueous solutions of glucose and of protein hydrolysate (a commercial preparation made by the enzymic digestion of casein) was assessed in experiments similar to those just described

The therapies were 5 and 10 g glucose/100 ml HO, 25 and 5 g. protein hydrolysate/100 ml HO, the solutions were given intraperitoneally or by mouth, in 1 ml or 2 ml doses and at Z, Z+30, Z+60 or Z+120 min The median survival time was not significantly prolonged by these procedures and usually it was reduced Clark and Rossiter (1944) also found that carbohydrate feeding did not lessen the mortality among experimentally burned animals

MUSTARD GAS INTOXICATION

The literature on human cases of mustard gas intoxication does not contain any clear-cut evidence as to the existence of a "shock syndrome" in this condition Hermann (1918) has reported a condition of "shock" in some of the severe cases which he saw, but most accounts are silent on this subject In animals (goats, dogs, rats) Cameron (1941) has described a haemoconcentration (as evidenced by increased haemoglobin and red blood cell estimations) in the first 24-48 hours after skin contamination, the changes were less marked in the rabbit Cameron and Courtice (1942) have also described the later appearance of persistent diarrhoea and salivation leading to dehydration. loss of weight, and circulatory failure Smith et al (by communication in 1943), in a study on the toxic action of the β -chloroethyl vesicants in dogs, have also remarked that death is due to "anoxia" of the respiratory centres owing to peripheral circulatory failure brought on by a reduction in blood volume, which is in turn attributable to the loss of protein, electrolyte, and water through vomiting and diarrhoea, supplemented by a loss of red cells through unidentified

channels The total circulatory plasma protein is reduced, but more water than protein is lost, so that the concentration of plasma protein rises. There is a reduction in the extra-cellular fluid, circulating plasma volume, plasma chloride, and body weight, the total circulatory red cell volume is variably reduced. As the investigators have pointed out, the

"progressive, unarrestable, and irremediable oligaemia with irreversible circulatory failure presents a close parallel to the sequence observed in dogs and man suffering fatal fire burns, haemorrhage and other forms of shock, and it is to be anticipated in men suffering severe vesication from mustard gas burns of the skin, and in whom a variable degree of systemic intoxication is superimposed on the loss of protein, the electrolytes and water through the vesicated areas"

In view of these reports of early haemoconcentration and later dehydration and electrolytic loss in mustard gas contaminated animals, it was decided to assess the value of sodium chloride administration in such animals. Three species have been studied, viz, the rat, rabbit, and goat

Rats—Rats (ca 150 g) were used first as the test animals, in all experiments they were contaminated by applying 10 mg/kg of mustard gas in 1 per cent (w/v) alcoholic solution to the shaven area of the back. Precautions were taken to prevent the rats licking the contaminated area and ingesting the contaminant. The results are given in Table II

We can conclude that in mustard gas contaminated rats

- (1) Sodium chloride solutions are an effective treatment and the sooner they are administered the better
- (11) Although the difference is not statistically significant, sodium chloride is probably more effective when given by mouth than when given intraperitoneally
- (111) Sodium lactate is ineffective either by mouth or intraperitoneally (This is contrary to our experience in the treatment of lewisite intoxicated animals, and to Rosenthal's results with severely burnt mice. It emphasizes the possibility that different biochemical reactions may be involved.)
- (iv) Water produced no significant effect, which suggests that electrolyte loss is a more important factor than mere dehydration in the pathology of mustard gas poisoning

Rabbits —Rabbits were contaminated with 150 mg / kg of mustard gas (pure) on the shaven area of the back, licking of the area was prevented. Half the rabbits were treated with 150 ml (given in two doses

15 ml isotonic sodium lactate

EFFECT OF SODIUM SALT	EFFECT OF SODIUM SALT SOLUTIONS ON THE SURVIVAL OF RATS INTOXICATED WITH MUSTARD GAS								
Treatment	Time of administration after contamination -	Route	No of animals	No of deaths in 21 days	Per cent mortality				
Nıl			70	56	80				
15 ml 0 9% NaCl	At once	IP	20	6	30				
15 ml 0 9% NaCl	15 minutes	1 P	20	5 ~	25 60				
15 ml 0 9% NaCl	30 ,,	l P	20	12	60				
15 ml isotonic sodium lactate	15 ,,	1 P	10	9	90				
15 ml water	15 ,,	Oral	10	7	70				
15 ml 0 9% NaCl	15 ,,		20	6	30				
1 ml 15% NaCl	15 ,	,,	10	2	20				
1 ml 30% NaCl	15 ,,	,,	10	2	20				
15 ml isotonic sodium loctate	15 "	1 "	10	و ا	በ የ				

TABLE II

(1 P = intraperitoneal)

This table shows the mortality rate in untreated rats to be 80 per cent and in rats treated with sodium chloride 37 per cent (significant at P = 0.000,000,1)

of 75 ml at 2 and 4 hours after contamination) of 09 per cent NaCl by mouth, the other half were not treated The 28-day mortalities were

Treated rabbits -2/10—1 e, 20 per cent Untreated rabbits - 7/10-1e, 70 per cent

This reduction in mortality is significant at P=0.04, if we take into account that the difference is in the right direction. The incidence and severity of diarrhoea and salivation and the loss of weight were much less in the treated than in the untreated group

Goats—The shaven backs of goats (circa 30 kg) were contaminated with 35 mg/kg mustard gas. The 09 per cent NaCl solution was given subcutaneously or by mouth, 1 litre at 2 hours and 1 litre at 4 hours after contamination The 28-day mortalities for the goats in the various groups were 7/10 in untreated goats, 7/10 in goats receiving NaCl subcutaneously, and 6/10 in goats given the NaCl by mouth Hence the administration of sodium chloride did not reduce the mortality from mustard gas contamination in Given subcutaneously, the saline solution caused massive pulmonary oedema in some of the goats (4/10), whereas by mouth it led to an increased incidence of diarrhoea (5/10, cf 2/10 in the untreated group)

Therefore, the order of efficacy of sodium chloride solutions in the treatment of the oligaemia of mustard gas intoxication in the three species tested descends as follows rats, rabbits, and goats This is also, in our experience, the descending order of the ability of the different species to tolerate large quantities of sodium chloride solution

SUMMARY

In confirmation of previous workers, it has been found that sodium chloride solutions increase the survival time and reduce the mortality rate of mice suffering from shock induced by immersion in hot The best results were obtained by giving 1 ml /0 45 per cent (w/v) sodium chloride solution 30 min after burning mice weighing about 20 g Glucose and protein hydrolysate solutions did not increase the survival time in our experiments

These results suggested the use of sodium salt solutions in the treatment of mustard gas intoxication This form of therapy was highly effective in rats and rabbits, it was ineffective in goats

The work on mustard gas intoxication was done at the Chemical Defence Experimental Station, Porton, and I am indebted to the Chief Scientific Officer, Ministry of Supply, for permission to publish these results

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THE CONSTITUTION OF PHOTOSTILBAMIDINE, THE IRRADIATION PRODUCT OF STILBAMIDINE

BY

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Stilbamidine (4 4'-diamidinostilbene β -hydroxyethanesulphonate) has proved an active therapeutic agent in a number of protozoal diseases During the treatment of Gambian sleeping sickness, Bowesman (1940) noted that solutions which had been made up for more than six hours were more toxic than those freshly prepared (1942) suggested that the poisonous effects noted by him during the treatment of kala-azar in the Sudan were due to the use of old solutions of stilbamidine which had become toxic. Fulton and Yorke (1942) investigated the matter experimentally and showed that the increased toxicity was the result of photochemical change in the stilbamidine molecule Similar changes were observed by Fulton (1943) in other diamidines with an unsaturated linkage The saturation of the ethylene linkage under these conditions was indicated by the spectrographic observations of Goodwin (1943) Barber, Slack, and Wien (1943) came to the conclusion, without rigorous proof, that the irradiation product- from stilbamidine was 4 4'-diamidinohenylbenzylcarbinol formed by the addition of water to the ethylenic bond Henry (1943) suggested, as an alternative to carbinol formation, a number of other changes which may occur in solutions of stilbamidine exposed to light including dimerization, partial or complete hydrolysis of the amidine groups, and formation of cis-stilbamidine The two latter suggestions could not be confirmed by Fulton and Goodwin (1946) In a later paper the same author (Henry, 1946) stated that aqueous solutions of trans-4 4'-diamidinostilbene undergo two concurrent changes—dimerization and cistrans isomerism—when irradiated in tropical sun-The proof of the dimeric nature of the saturated compound formed was arrived at from the kinetics of the photochemical reaction nature of this saturated compound has now been Ciamician and Silber (1902) described the formation of a dimer from stilbene when

exposed for two and a half years to sunlight in benzene solution. Their work was confirmed by Stobbe (1914) who showed that the dimer could be isolated after a few days' exposure to sunlight. We have obtained the dimer described by them by irradiating stilbene in benzene with the light of a mercury vapour lamp and have used it as a reference compound to prove that the saturated product obtained from stilbamidine by irradiation is 1 2 3 4-tetra-(4'-amidinophenyl)-cyclobutane, which we have, for simplicity, called photostilbamidine

Our thanks are due to May and Baker, Limited, for the gift of 50 g stilbamidine with which these experiments were conducted

EXPERIMENTAL

In the preparation of photostilbamidine, trans-4 4'diamidinostilbene β -hydroxyethanesulphonate (50 g) in 1 per cent (w/v) solution in distilled water was exposed in 500 cc volumes in pyrex conical flasks to direct sunlight When freshly prepared, the solutions, before irradiation, gave rise immediately to a bulky precipitate on mixing with an equal volume of dilute sulphuric acid After exposure of the solutions for 2 days, precipitation with sulphuric acid no longer occurred at once At the end of one week's exposure to sunlight an equal volume of 2N-sulphuric acid was added to the contents of the flasks and on standing a crystalline precipitate separated The yield of this first fraction after drying in air was 33 g The solution was concentrated to 1,800 c c under reduced pressure at a temperature below 50°C, and on standing a further crop of crystals (19 g) was obtained solution was partly neutralized with anhydrous sodium carbonate, and mixed with 600 cc of a saturated solution of sodium picrate, a bulky precipitate containing sulphate ions was formed which was collected, air dried, and triturated with 16 per cent aqueous hydrochloric acid The free picric acid was collected and washed with hydrochloric acid The combined filtrates were extracted with ether, and heated with norite to remove all traces of picric acid. A further

20 g of photostilbamidine as sulphate was obtained on concentration of the solution The absorption spectra of the various crystalline fractions and solutions were obtained during the course of the Apart from the presence of small preparation amounts of unchanged stilbamidine, amounting to approximately 5 per cent of the original, the only other substance detected was photostilbamidine. The latter, as sulphate, was purified for analysis by recrystallization from water in which it was sparingly soluble, it crystallized in short rods with terminal branching, and melted between 280 and 290° fied photostilbamidine sulphate in solution showed only the faintest fluorescence in ultra-violet light in contrast to the marked fluorescence of solutions of stilbamidine The air-dried product was analysed C, 445, H, 6.2, N, 126, H₂O, 157 C, H, N, 2H-SO, 8H, O requires C, 442, H, 60, N, 129, H,O, 166%) Photostilbamidine sulphate dissolved in water and treated with the theoretical amount of barium chloride gave a very soluble crystalline dihydrochloride When the solution of the latter was mixed with ammonium nitrate in excess, a crystalline nitrate was deposited Recrystallization from water gave compact crystals, mp 288° (decomposition) (Found in air-dried solid C, 466, H, 53, N, 201, H₂O, 57 C₃₂H₃N₈, 4HNO₃, 3H₂O requires C, 460, H, 51, N, 201, HO, 6.5%)

Hydrolysis of stilbamidine and photostilbamidine sulphate

Photostilbamidine sulphate (13 g) was mixed with potassium hydroxide (5 g), water (5 cc), and absolute alcohol (45 cc), and boiled for 22 hours with the addition of water (15 cc) at the start of each day to dissolve some of the salts present. An equal volume of water was then added to the mixture and the alcohol distilled off After removal of a small amount of insoluble material, the filtrate was acidified and yielded a gelatinous precipitate which became granular when heated on the water bath collected, thoroughly washed with water, and redissolved in sodium bicarbonate solution, the solution was filtered from inorganic material and acidified The product was again dissolved in aqueous sodium bicarbonate and reprecipitated The yields of acid in two experiments each from 67 g of starting material were 380 g and 385 g, after drying at 90° product was nitrogen-free and required the theoretical amount of base for neutralization On crystallization from glacial acetic acid, a small amount of insoluble material, possibly stilbenedicarboxylic acid, was re-After three recrystallizations, 0.39 g of the product required 35 cc of boiling glacial acetic acid for solution and gave 0.28 g of a colourless product. Two forms of crystal were present, a needle-shaped variety being preponderant with some hexagonal plates The tetra-acid was also soluble in ethyl alcohol acetone and ethyl acetate and did not melt below 310° (Found in solid dried at 90° C, 694, H, 51, loss at 110°, 31 C, H₂₄O₄, HO requires C, 693,

H, 47, H₂O, 3.2%) The methyl ester was prepared by the action of an excess of diazomethane in ether on 01 g of purified acid in 10 cc of methyl alcohol, after leaving overnight a clear solution was obtained, and when the solvent was removed an oil separated which soon solidified to a colourless solid. It crystallized from methyl alcohol in every small plates, mp 166° (Found in air-dried material C, 729, H, 54 C₂₈H₂₃O₈ requires C, 730, H, 54%)

The hydrolysis of stilbamidine was found to be a much more difficult process When 4 4'-diamidinostilbene dihydrochloride (25 g), potassium hydroxide (10 g) in water (10 cc) and absolute alcohol (90 cc) were refluxed for 20 hours in the dark, only 130 mg of an acid were obtained by the procedure described above In another experiment the same material (2.5 g.). sodium hydroxide (5 g) in water (10 cc) and ethylene glycol (40 cc) were refluxed for 9 hours The insoluble material present was removed and the filtrate concentrated under reduced pressure, the sodium salt obtained was collected, redissolved in water, and by the usual procedure yielded 054 g. acid, which was nitrogen-free, was insoluble in all common organic solvents and could not be crystallized. The ammonium salt, however, was very characteristic and crystallized readily in sheaves of leaflets. The solid first collected after alkaline hydrolysis weighed 1 57 g and contained nitrogen Hydrolysis of this material was effected by refluxing with 60 per cent sulphuric acid (25 cc) for _ 7 hours and then for a further 7 hours after addition of an equal volume of sulphuric acid of the same On cooling the mixture was poured into water, the solid collected, and after purification by solution in sodium bicarbonate solution the yield of air-dried material obtained on acidification was 0.9 g and had similar properties to the product of alkaline hydrolysis

Esterification could not be satisfactorily effected by refluxing the acid (0.5 g) with methyl alcohol (20 cc) and concentrated sulphume acid (2 cc) on the water bath for 4 days The desired product was, however, i obtained by allowing suspensions of the acid, from both alkaline and acid hydrolysis, in methyl alcohol and ether to react with diazomethane. The bulk of the reaction product was very sparingly soluble and separated as small rhomboidal plates, a further small amount was obtained by concentration of the mother This methyl ester was very sparingly soluble liquors in boiling methyl alcohol but crystallized well from dioxane in glistening fasciated laths, the addition of methyl or isopropyl alcohol to this solvent favoured quantitative separation The ester from both products had mp 234° Meyer and Hofmann (1917) gave the mp of dimethyl pp'-stilbene-dicarboxylate as 226-227° and Hager et al (1944) recorded the mp as 227-228°

Decarboxylation of 1 2 3 4--tetra-(p-carboxy-phenyl)-cyclobutane Isolation of distilbene isolatilbene and stilbene

The tetracarboxylic acid (0.5 g) was mixed with quinoline (10 cc) and copper bronze (0.5 g) and the

mixture boiled for 7 hours on a sand-bath Evolution of carbon dioxide was slow at first but soon increased as the held dissolved. On cooling, the mixture was poured into excess of 2N-hydrochloric acid and extraction of the product was effected by several portions of ether The combined ether extracts were washed in turn with N-hydrochloric acid, sodium bicarbonate solution, and finally water On removal of the solvent, 030 g of a crystalline material was It was recrystallized from ether and separated as a mixture of fine prisms and colourless plates The experiment was repeated with 15 g acid, yielding 10 g of product, which when refluxed with ether did not all go readily into solution. The less soluble portion was collected separately and crystallized from this solvent in the form of fine prisms, mp 163° (Found C, 933, H, 67 Calc for C, H₂₄ C, 933, H, 67% MW in camphor, 292 Calc 360) The MW in camphor, 292 Calc, 360) The properties of this hydrocarbon agree with those described for distilbene by Ciamician and Silber and it must be 1 2 3 4-tetraphenylcyclobutane more soluble portion separated on concentration of the first mother liquors, as colourless plates were shown by mp and mixed mp with a pure specimen to be stilbene. The yield of stilbene was approximately 1/5 that of the other product decarboxylation under the same conditions was contunued for 10 hours with 30 g acid obtained from carefully purified photostilbamidine sulphate the product weighing 2.15 g was highly coloured and contained a small amount of tarry material It was also much more soluble in ether, and from this solvent large crystals of stilbene separated first. The only other product obtained was that melting at 163° seemed that some decomposition had occurred during this longer decarboxylation and stilbene was present in relatively greater quantity than in the preceding A final decarboxylation with similarly purified material (10 g) lasting 4½ hours yielded 068 g of product By fractional crystallization from ether a large crop of the product mp 163° was obtained The next fraction yielded hexagonal plates which when crystallized from ether or methyl alcohol melted at 149° in a total yield of approximately 70 mg The only other product obtained was a few milligrams of stilbene It appeared, therefore, that the yield of stilbene increased with increase of the reaction time, and the hydrocarbon mp 149° could not be isolated when the reaction was continued for more than than The new hydrocarbon could be crystallized from ether or methyl alcohol and separated from the latter solvent in small tablets isomeric with distilbene, the name isodistilbene is proposed for it (Found C, 927, H, 68 requires C, 933, H, 67 MW in camphor, 307 Calc, 360)

Stilbene-4 4'-dicarboxylic acid on decarboxylation in the presence of quinoline and copper bronze for 4½ hours gave a theoretical yield of hydrocarbon Careful fractionation from ether and absorption on alumina failed to reveal any other hydrocarbon than

stilbene When stilbene and the hydrocarbon mp 163° were respectively heated with quinoline and copper under the same conditions, each substance was recovered unchanged from the reaction mixture

Fractionation of the acid product from photostilbamidine

In order to throw light on the presence of stilbene obtained along with the hydrocarbon mp 163° in all the decarboxylation experiments, the photostilbamidine used in the preparation of the acid was examined for purity. In water it showed fluorescence similar to that of a weak solution of stilbamidine and spectrophotometric determinations indicated that about 5 per cent of this substance was present Accordingly 144 g of the original photostilbamidine was recrystallized from water and 062 g of very sparingly soluble material with all the properties of stilbamidine sulphate, including characteristic absorption spectrum, was collected Further light on the homogeneity of the original photostilbamidine was sought by an examination of the acid obtained from This acid was subjected to a fractionation process depending on the different acidities of the acids (cf King and Ware, 1941) For this purpose 3 62 g of acid were accurately neutralized with 27 cc of approximately N-sodium hydroxide in a total volume of about 150 cc of aqueous solution The solution was then saturated with ether and a layer of 50 cc of the same solvent added 27 cc of N-hydrochloric acid was then added slowly with shaking the precipitated acid in this first fraction remained undissolved by the ether Ten further similar volumes of hydrochloric acid were added in turn, and the liberated acid dissolved in the freshly added ether layer in each case on shaking After removal of solvent the weights of acid in grams obtained from each fraction in turn were as follows 005 (insoluble) plus 006 (soluble), 017, 033, 040, 050, 045, 044, 044, 044, 015 All the fractions, in methyl alcohol, were methylated by means of diazo-methane From each, the methyl ester already described of mp 166° was obtained Only the insoluble part of the first fraction yielded the methyl ester of stilbenedicarboxyle acid, m p 234° The amount of this acid present was therefore probably less than 2 per cent and this amount failed to explain the presence of stilbene in the relatively large amounts found after decarboxylation

Irradiation of Stilbene

In contrast to the rapidity and completeness with which photochemical change occurs in stilbamidine solutions, the reaction in stilbene solutions occurs relatively slowly and incompletely. The formation of a distilbene mp 163° was described by Ciamician and Silber (1902) who exposed stilbene, in benzene solution, for 2½ years to sunlight. The same product was obtained by Stobbe (1914) after 3 days' exposure, but the percentage yield obtained was not indicated

by any of these authors We exposed 1 g of stilbene in 10 cc of pure benzene to sunlight (not intense) for six weeks in a sealed tube but failed to isolate any of the dimerization product The same solution was further exposed in a quartz cell 1 cm deep to light of wavelength 250 mµ for 4 hours again without success On further irradiation with the light from a mercury vapour lamp for 14 hours, the solvent was removed in vacuo and the crystalline residue redissolved in ether which was then concentrated prisms weighing 60 mg, readily distinguishable from the plates characteristic of stilbene, were the first to separate and a further smaller crop of prisms was On purification from ether the prisms melted at 163°, and from their properties, including stability to permanganate, non-absorption of bromine and unchanged melting point on admixture, appeared to be the same substance as that described by the above authors There was no change in melting point on admixture with the decarboxylation product mp 163° described above The substance produced by the irradiation of stilbene and the decarboxylation product were shown to be identical by means of x-ray crystallographic analysis (Fulton and Dunitz, 1947) and by comparison of their absorption spectra (Good-

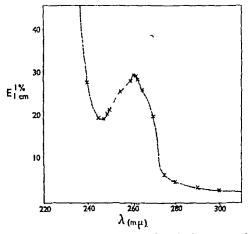


Fig 1—Absorption spectrum of 1 2 3 4-tetraphenylc) clobutane in cyclohexane

win, 1947) in cyclohexane (Fig. 1) Theoretically the position of the absorption band and the intensity measured as $E_{1\ cm}^{1\%}$ should be very similar to those for toluene, and this was found to be the case

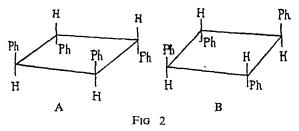
		max	E 1%
Compound mp	163°	261 mμ	29 9
Toluene		262 mu	32.7

When a stilbene solution in benzene was irradiated by the light of a mercury vapour lamp for 30 hours there was obtained besides unchanged stilbene and the hydrocarbon m.p 163° a substance in yield of only a few milligrams, which crystallized in colourless needles from ether and had mp 213° Analysis indicated that it contained oxygen Because of the extremely small amount available its nature could not be determined. In another experiment in which 135 g stilbene in benzene was exposed under the same conditions the yield of hydrocarbon mp 163° was 240 mg.

DISCUSSION

On exposure of stilbamidine solutions to sunlight the only product obtained, apart from small amounts of unchanged material, was photostilbamidine, isolated as sulphate We now have clear chemical evidence that 'photostilbamidine is a dimerization product of stilbamidine Analyses of photostilbamidine sulphate and nitrate show that the elements of water are not involved in the change of stilbamidine into photostilbamidine When photostilbamidine was hydrolysed an acid was obtained different from stilbene-p p'-dicarboxylic acid but of the same fundamental com-It gave a methyl ester, mp 166° different from the methyl ester of stilbene-p p'- 3 dicarboxylate which has mp 234° On decarboxylation of the acid from photostilbamidine, a hydrocarbon, C₂₈H₋₄, mp 163° was obtained identical with distilbene first isolated by Ciamician and Silber from the irradiation of benzene solutions of stilbene A second isomeric hydrocarbon isodistilbene mp 149° was found in one decarboxylation experiment

If during the irradiation of trans-stilbamidine the activated molecule retains its trans-structure, combination of two activated trans-molecules can give rise to two isomeric products which on hydrolysis and decarboxylation should yield the two tetraphenylcyclobutanes A and B



If however the activated molecule passed through or into a cis-form owing to free rotation, combinations involving cis-forms would give rise in addition to two other isomeric hydrocarbons

It has been shown (Fulton and Dunitz, 1947) that the distilbene, mp 163° is represented by A with a centre of symmetry. The spatial configuration of the isomeride, isodistilbene, mp 149° has not yet been determined, but the probability is that

it has structure B Since photostilbamidine in all experiments gave the hydrocarbon A, usually as the major component, the structure of photostilbamidine is that shown in A with four amidine groups in the p-positions of the phenyl groups

Since isodistilbene was isolated on one occasion it is possible that photostilbamidine sulphate contains a component corresponding to structure B The acid obtained by hydrolysis of photostilbamidine, on crystallization from acetic acid showed inhomogeneity of crystal form and on decarboxylation under milder conditions gave two isomeric hydrocarbons apart from stilbene the other hand, careful fractionation of the acidic product obtained on hydrolysis of photostilbamidine failed to show the presence of any isomeric tetracarboxylic acid since all the fractions gave the same methyl ester mp 166° The most likely explanation of this is that the isomeric tetracarboxylic acid which gives rise to the hydrocarbon, mp 149° must have been eliminated in the numerous purification stages involved in the preparation of the tetra-acid from photostilbamidine before subjection to decarboxylation

The formation of stilbene in the decarboxylations of the tetra-acid is definitely not due to the presence of stilbene-dicarboxylic acid as an impurity Stilbene arises from very specially purified photostilbamidine and its proportion increases with the duration of the decarboxylation process

SUMMARY

Irradiation of a solution of *trans*-stilbamidine in sunlight gave a substance which on hydrolysis to the corresponding acid, followed by decarboxyla-

tion, yielded a hydrocarbon m⁻p 163° identical with that obtained by irradiation of stilbene. The hydrocarbon previously described as di-stilbene has now been shown by x-ray crystallographic analysis to be 1 2 3 4-tetraphenyl-cyclobutane, containing a centre of symmetry. A second isomeric hydrocarbon mp 149° was also obtained in the same series of reactions, but its molecular configuration has not yet been determined. The isolation of these two hydrocarbons is in agreement with the view that stilbamidine undergoes dimerization on irradiation of its solutions.

I wish to record my thanks to Dr Harold King for generous help during the course of this investigation, and to Mr T W Goodwin for the determinations of the absorption spectra

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THE EFFECT OF INJECTED SOLUTIONS ON THE CELL CONTENT OF THE CEREBROSPINAL FLUID

BY

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An account has been given in an earlier paper (Bedford, 1946) of the effect of the introduction of isotonic sodium chloride solution into the cisterna magna on the cell content of the cerebrospinal fluid of dogs anaesthetized, with "nembutal" (Abbott) The duration of these experiments was approximately five hours morphonuclear leucocytes, generally unaccompanied by other reactive cells, were present at the end of nine out of thirteen experiments introduction under the same conditions of Ringer's solution (Dale's formula) or of distilled water did not cause reactive cells to appear in the cerebrospinal fluid. In the experiments now reported, the changes in cell count have been investigated 24 hours after the introduction of an isotonic solution of sodium chloride into the cisterna magna. and a comparison has been made with the effects produced after a similar period of time by solutions of procaine, amylocaine, and amethocaine hydrochlorides A study has also been made of the effect of simple puncture of the cisterna magna. the introduction of distilled water and of Ringer's solution

Experimental procedure

Except for slight modifications, the experimental procedure was similar to that used in earlier experiments (Bedford, 1946). In this series of experiments the animals were anaesthetized with ether. They were allowed to recover from the anaesthetic after the solution under investigation had been introduced and the needle withdrawn. It was necessary, however, after the introduction of local anaesthetics to maintain respiration with the pump until the onset of spontaneous respiration. The animals were again anaesthetized with ether twenty-four hours later and

a sample of cerebrospinal fluid removed for cytological examination The films were fixed by exposure to jodine vapour after the technique of Kubie and Smith (1925) and lightly stained with methylene The use of this technique has greatly facilitated cellular differentiation. In the present series of experiments a finer needle has been used for cisternal puncture, as a result, detached mesothelial cells have been observed only on rare occasions in specimens of cerebrospinal fluid, and the possibility of postoperative leak from the subarachnoid space has been reduced The puncture was made without previous incision directly through the shaved and sterilized skin An experiment was discontinued if the initial sample of cerebrospinal fluid contained cells of any kind The solutions were made up with freshly distilled water from an all-glass still Although the individual solutions were not tested for the presence of pyrogens, repeated examination of distilled water from this still has never revealed their presence. The solutions were sterilized, without delay in an autoclave at a pressure of 12 lb per square inch for half an hour Ringer's solution was boiled for 20 min at atmospheric pressure Care was taken to sterilize the solutions in alkali-free glass containers. A constant volume (15 cc) of solution, at room temperature, was introduced into the subarachnoid space throughout the experiments

RESULTS

The effect of ether anaesthesia

Four dogs were anaesthetized with ether, one for a period of 15 minutes, two for 30 minutes, and one for one hour. The ether was administered by means of a pump through a catheter, introduced into the trachea. In all four dogs, samples of cerebrospinal fluid removed 24 hours later under ether anaesthesia were free from cells

The effect of simple puncture and of distilled water

The effect of simple puncture of the cisterna magna was studied in ten dogs. After introduction, the needle was allowed to remain in situ with the stilette inserted for ten minutes. At the end of this period, the needle was withdrawn and the animal allowed to recover. The results obtained 24 hours later are summarized in Table I,

TABLE I

THE EFFECT OF SIMPLE PUNCTURE OF THE CISTERNA MAGNA
AND OF DISTILLED WATER ON THE CELL CONTENT
OF THE CEREBROSPINAL FLUID

Simple puncture		Distilled water (1 5 c.c.)		
Wt of dog m kg	White cells per cu.mm CSF after 24 hours	Wt. of dog m kg	White cells per cu mm CST after 24 hours	
11 0 8 0 7 5 11 0 8 0 8 6 12 5 7 5 8 0 6 5	40 70 60 70 20 40 12 40 0	10 0 9 0 8 5 10 0 7 5 6 0	480 450 450 345 420 400 —	
Mean ± S D 36 ± 8			424 ± 19	

where it will be noticed that white cells were present at the end of all but one experiment. The average number of cells per cumm of cerebrospinal fluid was 36. The effect of the introduction of distilled water was studied in six animals. The results are summarized in Table I, where it will be seen that the average number of white cells at the end of the experiment was 424, and the standard deviation of the average 19

The effect of isotonic sodium chloride and of Ringer's solution

The effect of isotonic sodium chloride solution (09 g NaCl/100 cc) was studied in nine experiments and that of Ringer's solution (Dale's formula) in six experiments. The pH values of the solutions after sterilization were 68 and 74 respectively, as determined by indicators. The results of the experiments are summarized in Table II.

The effect of solutions of procaine, amylocaine, and amethocaine hydrochlorides

The effect of procaine was studied in ten experiments, five experiments were performed with

TABLE II

THE EFFECT OF ISOTONIC SODIUM CHLORIDE SOLUTION AND OF RINGER'S SOLUTION (DALE'S FORMULA) ON THE CELL CONTENT OF THE CEREBROSPINAL FLUID AFTER 24 HOURS

Normal Sodium Chloride Solution (1 5 c c)		Ringer's Solution Dale's Formula (15cc)		
Wt of dog in kg	White cells per cu mm CSF after 24 hours	Wt of White cells dog cu mm CS in kg after 24 hou		
7 5 5 8 0 7 0 8 5 7 0 8 5 7 5	1,000 750 1,500 750 700 1,500 800 1,600 1,000	9 0 6 0 8 0 7 0 7 5 6 5	740 490 720 690 470 680 —	
Mean ± S D 1,067 ± 109			632 ± 49	

amylocaine and a similar number with amethocaine Procaine and amylocaine were administered in a 1 per cent (w/v) concentration in distilled water. The depressant action of amethocaine on respiration was so powerful and prolonged that it was found convenient to use a solution not stronger than 0.25 per cent, even after the introduction of a solution of this concentration, respiration was frequently paralysed for half an hour or longer. The pH values of the procaine, amylocaine, and amethocaine solutions after sterilization were 5.9, 5.9, and 6.0 respectively. The results of these experiments are summarized in Table III.

It will be seen that the average number of cells per cumm of cerebrospinal fluid at the end of the experiments was approximately the same with all three drugs

DISCUSSION

It would seem from the above experiments that the introduction into the subarachnoid space of isotonic sodium chloride solution, procaine, and amylocaine hydrochlorides in 1 per cent (w/v) and amethocaine hydrochloride in 025 per cent (w/v) concentration in distilled water excites reactions of approximately equal intensity as determined by the number of white blood corpuscles in the cerebrospinal fluid after 24 hours Ringer's solution caused a more intense reaction than distilled water, but not so powerful as that of isotonic sodium chloride solution, it occupied a position intermediate between the two The standard deviations of the averages obtained in these experiments indicate that the above differences in

TABLE III

THE EFFECT OF PROCAINE, AMYLOCAINE AND AMETHOCAINE HYDROCHLORIDES ON THE CELL CONTENT OF THE CEREBROSPINAL FLUID

1% (w/v) Pr	ocame (1 5 c c)	1% (w/v) An	1% (w/v) Amylocaine (1 5 c c)		nethocaine (1 5 c c)
Weight in kg	White cells per cu mm CSF after 24 hours	Weight in kg	eight in kg White cells per cu mm CSF after 24 hours		White cells per cu mm CSF after 24 hours
6 5 10 5 12 0 8 0 10 0 7 0 8 5 7 5 9 0 8 5	1,340 840 1,500 810 900 1,200 750 825 1,180 1,010	6 2 12 0 12 5 7 5 12 0	1,600 800 810 1,090 700 — —	8 5 7 5 8 0 9 5 6 5 — — —	670 1,490 730 1,360 1,500
Mean ± S D	1,035 ± 81		1,000 ± 163		1,150 ± 185

intensity of reaction are statistically significant. The lack of correlation between the weight of the animal and the reaction provoked by a constant volume of solution was unexpected. It can, however, be accounted for in several ways The dogs used in these experiments were mongrels, little selection has been possible and consequently marked variation was noticed in the size and shape of the cisterna magna of dogs of the same weight. Variations will accordingly occur in the degree of dilution of solution immediately after introduction and in the rate at which it leaves the cisterna An important cause of lack of correlation between body weight and reaction to a given volume of solution is leakage through the puncture hole after withdrawal of the needle It has been possible to 'demonstrate that the puncture hole through the dura and the arachnoid does not always close after withdrawal of the needle, the longer the needle remains inserted, the more likely is leakage to occur A leaking puncture hole can produce two important results if the pressure in the cisterna magna is high, as normally occurs when the animal is inclined towards the "headdown-tail-up" posture, fluid escapes, often at a considerable rate from the cisterna magna into the extradural region On the other hand, if the animal is inclined towards the "head-up-taildown" posture the pressure in the cisterna magna falls and aspiration may take place from the The fluid aspirated generally extradural region consists of blood and tissue fluid, which are known (Hammes, 1944) to have a marked irritant action on the meninges These phenomena have frequently been demonstrated on the living animal during the course of the experiments. Their occurrence can be avoided to a certain extent by the use of a fine needle and by careful avoidance of any disturbance of the needle after introduction.

The cells present in the cerebrospinal fluid at the end of the experiments consisted almost entirely of polymorphonuclear leucocytes, in many instances they were the only cells detected Lymphocytes rarely formed more than 3 per cent of the total white cells

It is improbable that the cellular reaction provoked by local anaesthetics is a pH response Experiments in vitro have shown that the solutions rapidly assumed the pH of the cerebrospinal fluid on the addition of a relatively small volume of the latter fluid. Injected solution, withdrawn a few seconds after introduction into the cisterna magna, was generally found to have acquired the pH of cerebrospinal fluid.

In spite of technical difficulties, the experiments would appear to demonstrate that distilled water is a less powerful irritant to the meninges than isotonic sodium chloride solution and thereby to confirm the results of earlier experiments (Bedford, 1946), that isotonic sodium chloride solution, procaine, and amylocaine hydrochlorides in a 1 per cent (w/v) and amethocaine hydrochloride in a 0.25 per cent concentration in distilled water excite reactions of approximately equal intensity

The results of the experiments raise doubt as to the advisability of using simple sodium chloride in order to render isotonic solutions required for introduction into the subarachnoid space

SUMMARY

- 1 A study has been made of the cell content of the cerebrospinal fluid 24 hours after the introduction of procaine, amvlocaine, and amethocaine into the cisterna magna of dogs and the effects of these drugs have been compared with those produced by distilled water, isotonic sodium chloride and Ringer's solution over a similar period of time
- 2 Isotonic sodium chloride solution, 1 per cent procaine and amylocaine hydrochlorides and 0.25 per cent amethocaine hydrochloride in distilled water excited reactions of approximately equal
- intensity Ringer's solution caused a more intense reaction than distilled water, but less than that of isotonic sodium chloride solution
- 3 The results of these experiments raise doubt as to the advisability of using simple sodium chloride in order to render isotonic solutions required for introduction into the subarachnoid space

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ACTIONS OF BRITISH ANTI-LEWISITE (2 3-DIMERCAPTOPROPANOL)

BY

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Peters, Stocken, and Thompson (1945) and Waters and Stock (1945) described the effectiveness of 2 3-dimercaptopropanol (named British Anti-Lewisite or BAL by American workers) in countering the effects of arsenical poisoning Apart from the importance of having an efficient entidote to arsenical agents in chemical warfare, there arose the possibility of using BAL for the treatment of the toxic manifestations of arsenical drugs in therapeutics. After investigations of the protective action of BAL against arsenical poisons carried out in this country and at the same time those on lewisite by Harrison, Durlacher, Albrink, Ordway, and Bunting (1946) ne substance was used successfully by Carleton. ters, Stocken, Thompson, and Williams (1946) eru Eagle (1946) as an antidote in clinical cases of poisoning by such therapeutic agents as · Papharside

The protective action of BAL stimulated research into the mode of action of dithiols in detoxicating arsenic. Peters, Sinclair, and Thompson (1946) and Stocken and Thompson (1946) have shown that arsenic combines with the available sulphydryl groups in proteins and in this way interferes with the enzymes vital to cellular metabolism which depend for their activity on the presence of SH-groups, one such enzyme is pyruvate oxidase. A molecule of BAL contains two SH-groups and can thus re-activate enzymes which have been inhibited by arsenic (Stocken, Thompson, and Whittaker, 1947)

The nature of the reaction between arsenic and BAL made it seem probable that BAL would react similarly with other metals, and Gilman, Philips, Allen, and Koelle (1946) and Ginzler, Gilman, Philips, Allen, and Koelle (1946) showed that this was so with cadmium Braun, Lusky, and Calvery (1946) showed the same with antimony, bismuth, chromium, mercury, and nickel Lead and selenium were made more toxic by BAL, while thallium was not affected

The toxic properties of BAL were investigated by Durlacher, Bunting, Harrison, Ordway, and Albrink (1946) and Modell, Chenoweth, and Krop (1946) who stated that the LD50 in rabbits was 99 mg/kg and in cats 0 032 ml/kg. The chief symptoms of toxicity found were conjunctivitis, gastro-enteritis, tremors, and ataxia. Fatal doses caused convulsions and circulatory and respiratory failure. According to Modell, Gold, and Catell (1946) doses in excess of 3-5 mg/kg in man cause blepharospasm, flushes, and unpleasant sensations.

It was decided to investigate the action of BAL on the toxicity of several-metallic compounds deemed to be of clinical importance, viz.—mapharside, mercuric chloride, mersalyl, potassium antimony tartrate, lead acetate, sodium bismuth tartrate, sodium auro-thiomalate and chromium trioxide. In addition the effect of BAL on the physiological action of insulin was examined. As a preliminary step the toxicity of BAL itself was investigated.

EXPERIMENTAL

Methods - The effects of BAL were investigated by injection of freshly prepared watery solutions of the compound by various routes into mice, rats, guineapigs, and rabbits Rabbits anaesthetized with 25 per cent (w/v) urethane solution and cats anaesthetized with ether and chloralose (80 mg/kg iv) were used to determine the actions of BAL on the blood pres sure, heart (myograph), spleen volume (plethysmo graph and piston recorder), leg volume (plethysmograph and tambour), and respiration (stethographic lever) Isolated rabbit auricles, isolated perfused cat hearts, and isolated strips of gut and uterus from rabbit, cat, and guinea-pig were examined in the usual The vessels of the rabbit ear were perfused with saline according to the method of Gaddum and Kwiatkowski (1938), but as no recording apparatus was available the outflow was measured in drops per 15 sec using larger doses of drugs than would otherwise be necessary The method of investigating the effect of BAL on the diuretic activity of mersalyl was that

described by Burn (1937) for the assay of the antidiuretic potency of extracts of the posterior pituitary body

The LD50 of the metallic compounds was roughly determined by intra-peritoneal injection in small groups of mice, 200 male mice of 20-30 g weight were then taken in four groups of 50 and given graded doses of the metallic compound so as to cover an adequate range of toxicity. To half of each group of 50 BAL was then given in a standard dose of 40 mg/kg ip and the mortalities noted after 24 nours. Where the detoxifying action of BAL was such as to cause all the animals so protected to survive, the experiment was repeated with higher doses of metallic compound in the animals receiving protection. All injections with one metal were done at a single session. The LD50 was read from the plot of log dose and probit of lethality.

RESULTS

Toxicity of BAL

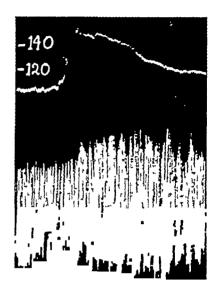
The LD50 of BAL dissolved in water was found to be 100 mg/kg for white mice injected intraperitoneally, which agrees well with the 0.8 mM/kg of Durlacher et al (1946). Three guinea-pigs given 150 mg/kg died, and three given 50 mg/kg survived, 12 rats survived 40 mg/kg 1p, and of six rabbits given 100 mg/kg 1v two died

In mice a lethal dose given intraperitoneally caused immediate weakness of the legs, analgesia was marked at this early stage, severe nipping of the tail being ignored, respiration was slow and laboured, but no tremors were seen, clonic and tonic convulsions followed, interrupted by periods of coma, death was accompanied by signs of asphyxia Sublethal doses produced marked ataxia and weakness but not convulsions In rabbits and guinea-pigs small doses (20 mg/kg 1p) produced blepharospasm and sneezing in 15 min, followed by tremor, weakness of the legs, and ataxia in 30 min to one hour Salivation was notable, urine and faeces were passed frequently, and the respiration was deep and hurried dose was lethal the tremor increased to generalized convulsions, respiration was markedly impaired, and death occurred in tonic convulsion Post mortem the liver, spleen, kidneys, and gut were congested and the lungs covered with small haemorrhages of the lungs revealed haemorrhagic exudates

Circulatory and respiratory effects

In rabbits anaesthetized with 25 per cent (w/v) urethane, BAL in a dosage of 0.5-10 mg/kg in saline had no appreciable effect on the blood pressure or respiration, whereas 40 mg/kg caused a sharp rise in blood pressure of some 20-30 mm Hg and a stimulation of respiration which was maintained for several minutes, this effect of small doses of BAL on the circulation

and breathing in the rabbit is illustrated in Fig 1 Larger doses (20-40 mg/kg) caused a transient rise in blood pressure and stimulation of respiration which was followed by a steady decline in pressure and failure of breathing. If the lethal dose was approached this course might be interrupted by convulsions, though usually the blood pressure declined to zero and the animal died



30

FIG 1—Rabbit & 2.4 kg Urethane 25 per cent 1 v and ether Upper record carotid blood pressure, lower record respiration (stethograph, inspiration down)
Time in 30 secs BAL (40 mg/kg 1 v) causes a rise in blood pressure and stimulation of respiration

quietly after a few final gasps. This was in contrast to the mode of death of the unanaesthetized animal which convulsed violently, but the lethal dose was no smaller in the unanaesthetized animal, nor was the toxic effect modified by atropine sulphate, vagotomy, or artificial respiration. The isolated auricles of the rabbit continued to beat well in the presence of BAL in high concentration (1 in 10,000). The perfused vessels of the rabbit ear were constricted by BAL. Single injections of 30 mg BAL in saline caused sharp but very transient vaso-constriction, constant perfusion with BAL (1 in 10,000) produced a reduction in flow.

In cats anaesthetized with ether, chloralose or nembutal, intravenous BAL invariably caused an abrupt fall in blood pressure accompanied by transient shrinkage of the spleen. If 20 mg/kg was given the blood pressure fell abruptly and was quickly but usually only partially restored,

though sometimes it rose again above the initial level for a few minutes The abrupt fall in pressure was accompanied by shrinkage of the spleen, or sometimes by dilatation, the phase of recovery, partial or complete, was accompanied by active splenic dilatation The leg was constricted and the heart unchanged in vigour and amplitude of beat so that splanchnic dilatation would appear to account for the early stages of the fall in pressure The relative degree of alteration in flow in the splanchnic and limb circulations accounts for the varying responses of the blood pressure Respiration was stimulated Within a few minutes a progressive fall in blood pressure and inhibition of respiration ensued Neither the abrupt nor the progressive fall in blood pressure was prevented by atropine sulphate (1.5 mg/kg), by bilateral vagotomy or by artificial respiration. so that there is no question of vagal inhibition playing a part in the initial fall in pressure nor of failure of oxygenation of the blood playing a part in the later fall in pressure After a few minutes, while the blood pressure was steadily falling, the spleen contracted maximally blood pressure at this stage could be temporarily restored by injection of adrenaline or infusion The restorative effect of saline soon of saline

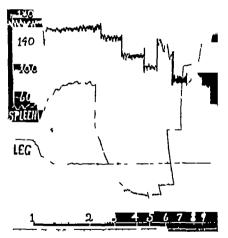


Fig 2—Cat, & 3 kg wt Ether and chloralose, 80 mg/kg Upper record carotid blood pressure, middle record splenic volume (plethysmograph and piston recorder), lower record leg volume (plethysmograph-tambour) Injection points and time in 30 secs are marked At 1, 40 mg BAL/kg was given iv at 11 43 a.m and 20 mg/kg repeated at 12 0 a.m., 1 56 p.m., and 2 10 p.m. (total 100 mg/kg), 10 0 ml saline at 12 21 temporarily restored the pressure. The time relationships are as follows: 1 = 11 43 a.m., 2 = 11 50 a.m., 3 = 11 54 a.m., 4 = 12 06 p.m., 5 = 12 24 p.m., 6 = 1 45 p.m., 7 = 1 53 p.m., 8 = 2 10 p.m., 9 = 2 20 p.m.

passed off, blood pressure fell progressively, the spleen dilated again, respiration became slow and gasping, the leg volume remained reduced, and if the dose was large enough death occurred despite artificial respiration. These changes are illustrated in Fig 2 When oxygenation was maintained by artificial respiration, the heart continued to act well and only failed 5-10 minutes after blood pressure had reached zero. The isolated perfused heart of the cat (Langendorff) showed no ill-effects from the injection of up to 50 mg BAL and the rate of coronary flow was un-The progressive fall in blood pressure with an active heart, in the presence of peripheral vasoconstriction, suggests a steady leakage of fluid from the circulation. The petechial haemorrhages seen on the lungs and liver indicate damage to capillaries and small vessels mean packed cell volume in five anaesthetized cats was 366 per cent (the blood having been spun for 25 min at 5,000 rpm), three hours after the administration of 100 mg BAL/kg and shortly before death took place it was 483 per cent, an increase of 32 per cent. This indicates a severe degree of haemoconcentration, despite the fact that' an average of 25 ml of saline and other solutions had been given intravenously in the course of the experiments, and it would appear to be the primary cause of death in anaesthetized animals, as Chenoweth (1946) indicated

The odour of BAL could be detected in blood, urine, tears, and expired air, and in the freshly cut organs after death

Action of BAL on the toxicity of metallic compounds Arsenic

The arsenical preparation used was mapharside, freshly made up and injected in 0.25 ml saline intraperitoneally into groups of white mice. The LD50 for mapharside alone was found to be 34.4 mg/kg, BAL had a strongly protective action, raising the LD50 to 350 mg/kg or tenfold. This action is illustrated in Fig. 3 and is in agreement with the findings of Stocken, Thompson, and Whittaker (1947) with rats

Twenty-four guinea-pigs of 350-400 g weight were given a daily injection of mapharside (15 mg/kg) subcutaneously, twelve were given in addition a daily injection of BAL (40 mg/kg) intraperitoneally. Eight of the guinea-pigs receiving arsenic alone had died with symptoms of restlessness, twitching, diarrhoea, wasting, and weakness between the fifth and tenth day of injection, when the remainder were killed. The guinea-pigs receiving BAL remained well and were sacrificed on the tenth day. Fresh specimens of liver,

kidney, spleen and gut were sectioned, stained, and examined The chief lesions caused by arsenic poisoning were necrosis of the liver lobules with much debris and exudate, severe disintegration of the glomeruli and tubules of the kidney, catarrhal changes in the mucous membrane of the gut and congestion of the spleen with necrotic changes in the pulp and nodules. These changes were prevented by BAL therapy, the most striking differences being in the kidney. It is evident that the combination of arsenic and BAL causes little tissue damage during its excretion.

Antimony

The preparation of antimony used was tartar emetic, which proved to have an LD50 of-562 mg/kg, 40 mg BAL/kg ip had a protective action on mice poisoned with this compound, raising the LD50 to 716 mg/kg. The results are illustrated in Fig. 3

Chromium

The preparation used was chromium trioxide, which had an LD50 of 663 mg/kg BAL (40 mg/kg 1 p) had a protective action against chromium poisoning, raising the LD50 to 858 mg/kg, this effect is illustrated in Fig 3

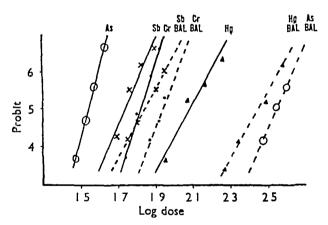


Fig 3—The protective action of BAL (40 mg/kg 1 p) on the toxicity of arsenic (mapharside) antimony (tartar emetic), chromium (chromic acid) and mercury (corrosive sublimate) in groups of white mice. The ordinates are probits of lethality, the abscissae logs of the dosage BAL decreased the toxicity of each metal, an effect illustrated by the shift of the lines to the right.

Twelve guinea-pigs were shaved and given 0.25 ml of 5 per cent (w/v) chromic acid solution intracutaneously as a wheal. After five days there were well-established circular ulcers at each site of injection with a black sloughing eschar about half an inch across and a raised red areola for a quarter-inch round the ulcer. The animals had recovered from the constitutional upset resulting from chromium absorption. At this point they were divided into two

groups and half the animals treated with a daily application of acriflavine ointment to the ulcers, the other half received the same ointment made up to contain 10 per cent of BAL The BAL ointment was kept cool between applications and retained its pungent odour to the end of the experiment ulcers treated with BAL became soft, filled in rapidly and had healed in 30 days, the ulcers treated with acriflavine alone were still indurated and approximately one quarter their original size after 40 days, when the animals were sacrificed It is suggested that treatment with BAL ointment, and, if necessary, systematically administered BAL, would be of value in industrial chrome ulceration

Mercury

The preparation tested was mercuric chloride, the LD50 of which was 120 mg/kg, BAL had a protective effect on animals poisoned with this salt, raising the LD50 to 281 mg/kg (see Fig 3) According to Long and Farah (1946) intravenous injection of BAL reduces the lethality of intravenous 'salyrgan' in mice, and protects the cardiovascular system of anaesthetized dogs from the toxic effects of this mercurial compound In the present work the LD50 of mersalvl BP solution was found to be 169 mg/kg ip in mice BAL (40 mg/kg 1p) immediately after the diuretic lowered the LD50 to 100 mg/kg mersalvl given intravenously the LD50 was 120 mg/kg and BAL (20 mg/kg) also given intravenously protected many of the mice from the violent convulsions caused by intravenous mersalyl and raised the LD50 to 165 mg/kg

The effects of BAL are discussed later, but some clarification was provided by the results of tests of the effect of mersalyl on water diuresis in groups of BAL (40 mg/kg ip) acted as an antidiuretic, suppressing urine for 3-4 hours Mersalyl (100 mg/ kg 1 p) caused immediate anuria and death followed after 48 hours, but if the two were given intraperitoneally within a few minutes of one another suppression was much less than with either alone, and the rats survived. In a double experiment using 16 rats the animals when watered and given subcutaneous saline had a diuresis of which the peak occurred after 65 min, after subcutaneous mersalyl (10 mg/kg) and saline the peak occurred at 72 min of mersalyl (10 mg/kg) and saline after one week gave a figure of 73 min, whereas mersalyl sc and BAL (40 mg/kg) 1p gave a figure of 90 min follows that mersalyl and BAL in large doses each have a delaying effect on the excretion of urine from normal rats and that given together they tend to cancel one another, with small doses this effect is not seen

Chronic mercurial poisoning was induced in 12 rabbits by giving them mercury perchloride (10 mg/kg) for ten days, and in 16 guinea-pigs by giving

50 mg/kg 1 p daily The animals so treated lost weight and developed diarrhoea and tremors and died, the guinea-pigs living for only 4 days, the rabbits for 12-16 days Half the rabbits were also given 2.5 mg BAL/kg sc daily, and half the guinea-pigs 40 mg/kg. The animals given BAL survived in good health until sacrificed Microscopy revealed marked necrosis of liver, kidney, and gut in the animals poisoned with mercury, and an absence of these lesions in the animals protected with BAL

Lead

The salt used was plumbi acetatis BP, the LD50 of which was 461 mg/kg 1p, BAL (40 mg/kg 1.p) had an additive effect on the toxicity of this substance, the LD50 falling to 416 mg/kg, this effect is shown in Fig 4

Gold

The salt used was sodium aurothiomalate and the LD50 found was 1096 g/kg ip, BAL had an additive effect on the toxicity of this compound, reducing the LD50 to 812 mg/kg, an effect shown in Fig 4 Successful clinical use of BAL in cases of gold poisoning is reported by Cohen, Goldman, and Dubbs (1947)

Bismuth

The salt tested was sodium and potassium bismuth tartrate, the LD50 of which was found to be 676 mg⁻⁷kg ip BAL had an additive effect on the toxicity of this compound, reducing the LD50 to 288 mg/kg, this effect is shown in Fig 4

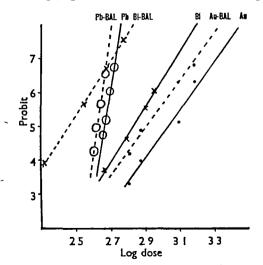


Fig 4—The action of BAL (40 mg/kg i p) on the toxicity of lead (lead acetate), bismuth (sodium and potassium bismuth tartrate) and gold (sodium auro-thiomalate) in groups of white mice. The ordinates are probits of lethality and the abscissae logs of dosage BAL increased the toxicity of each metal, an effect illustrated by the shift of the lines to the left.

Effect of BAL on the action of insulin

Barron, Miller, and Meyer (1947) state that the action of insulin (0 8~U/kg) is inhibited by BAL in doses of 0 1 g/kg iv Larger doses of BAL reduce the blood sugar of rabbits and prove fatal

In the present experiments a group of five rabbits about 20 kg weight were starved overnight and a blood sample of 05 ml drawn from the marginal ear vein of each rabbit and pooled in a heparinized tube. The animals were given 0.5 U/kg soluble insulin subcutaneously and pooled blood samples collected every hour After three days the experiment was repeated with the addition of 25 mg BAL/kg 1.p The mean blood sugar level after three hours had fallen by 75 per cent with the animals given insulin alone, whereas with insulin and BAL the level at three hours had fallen by 21 per cent. The insulin by itself gave a more precipitate and a more prolonged fall in blood sugar level than did the insulin in the same animals treated with BAL This inhibitor effect of BAL on the action of insulin is illustrated in Fig 5

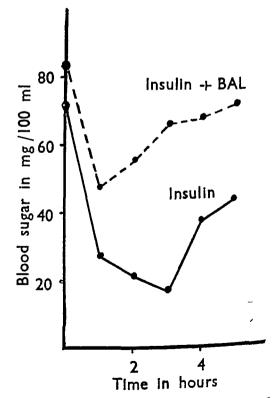


Fig 5 —The mean blood sugar curve in five rabbits after 0.5 U/kg soluble insulin s.c., and the effect of BAL (25 mg/kg i p) in reducing the effect of the same dose of insulin in the same animals.

DISCUSSION

It has been shown in the present work and in the work of others quoted that BAL is an effective antidote to poisoning by arsenic, mercury, antimony, chromium, nickel, and cadmium McCance and Widdowson (1946) found that the closely related BAL-glucoside promotes excretion of copper and zinc salts, and it has further been shown that iron and thallium are unaffected by thiol compounds whereas lead and selenium are rendered more toxic Bismuth and gold were found to be made more toxic to mice by BAL in the present work but according to Braun et al (1946) BAL rendered bismuth less toxic to rabbits and according to Ragan and Boots (1947) gold less These differing results together toxic to man with the apparently contradictory results obtained in the present work with mersalyl, and also the complex manifestations of the toxicity of BAL itself are attributable to the presence of sulphydryl groups in its molecule Webb and van Heyningen (1947) point out that BAL inhibits the activity of any enzyme which contains a heavy metal, capable of being linked with available SH, as prosthetic group to the protein moiety of the enzyme Such an activity would account for the widely differing phenomena of BAL poisoningconvulsive action on the CNS, drop in blood sugar, interference with respiration, and presumably also the damage to the small vessels which produces such marked alterations in the circula-The smooth muscle of peripheral vessels appears to be much more sensitive to BAL than the muscle of gut, uterus, the heart, or coronary arteries, and the differing initial response of the spleen and limb volumes to intravenous injection of BAL needs further examination

Gilman et al (1946) point out that with cadmium the compound formed in vivo by BAL is a soluble substance which proves on isolation to have a greater toxicity than cadmium chloride This increased toxicity is caused by increased pathogenicity to the kidney The arsenic, mercury, chromium, and antimony preparations used to determine the LD50 in mice are highly irritant to the peritoneum, quickly absorbed and cause gross renal damage on excretion, intraperitoneal treatment with BAL reduced the toxicity of these compounds and prevented visible damage to the kidney The products of linkages between BAL and these metals must be of such a nature that they are not toxic to the kidney on excretion The reduction of acute toxicity of the metallic compound may be due to production of a non-irritant compound by reaction with BAL

in the peritoneal cavity, and this compound may be absorbed more slowly and excreted in a non-toxic form. The salts of gold, bismuth, and lead which were used have a much higher LD50 than the group of metals discussed above, when given intraperitoneally. They are not so irritant as the former, they tend to be precipitated and to be absorbed slowly, and thus the cause of their acute toxicity is probably different from that of the others. BAL-metal complexes with these metals may be absorbed more quickly or be more toxic than the metallic salts themselves.

A point of importance is suggested by the finding of Gilman et al (1946) that the BAL-cadmium complex formed in vitro differs markedly from the complex formed in vivo the present work with mice the metallic salts were given intraperitoneally followed by the BAL at the same site If an in vitro type of precipitate were formed in the peritoneal cavity, this might well differ in stability, absorption rate, and toxicity from the type of compound formed in vivo in rabbits which were injected inframuscularly at different sites with bismuth and BAL by Braun et al (1946) or from that in man given gold and BAL at separate sites and times by Ragan and Boots (1947) The same phenomenon of formation of different mercaptides with different toxicities according to the conditions of reaction of the metal and the thiol compound may account for the opposite effects of BAL on the toxicity of mersalyl given intraperitoneally and intravenously

The many toxic properties of BAL and the variability of its effects indicate that some other related compound would be preferable for therapeutic use Danielli, Danielli, Mitchell, Owen, and Shaw (1946) give evidence that BAL-glucoside may be suitable

SUMMARY

British Anti-Lewisite (BAL) has an LD50 of 100 mg/kg 1p in white mice It causes conjunctivitis, ataxia, rapid and then impaired respiration and convulsions in small mammals In anaesthetized rabbits small doses (4 mg/kg) cause a temporary rise in blood pressure, but in cats only a fall in pressure is seen, this is considered to be due initially to splanchnic dilatation, but the main effect on the circulation of anaesthetized cats is a progressive fall in blood pressure despite constriction of the leg and spleen and an active heart Loss of fluid from the capillaries leading to haemo-concentration and a state of shock is held to be the cause of death in anaesthetized animals, whereas convulsion is the cause of death in intact animals

BAL has a protective action on white mice poisoned with arsenic, mercury, antimony, and chromium and a deleterious effect on mice poisoned with lead, gold, and bismuth It prevents or relieves tissue damage caused by chronic poisoning with arsenic, mercury and chromium BAL inhibits the action of insulin Some apparent anomalies in the detoxifying action of BAL are discussed.

A pure sample of BAL was obtained from the Ministry of Supply The expenses of the investigation were in part defrayed by a grant from the Rankin Research Fund, University of Glasgow, to one of us (J H), and in part by a grant from the Medical Research Council One of us (J D P G) holds an ICI Fellowship in Pharmacology, Umversity of Glasgow We desire to express our grateful appreciation of the interest taken in the work by the late Prof N Morris

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A STUDY OF ANTHELMINTHIC POTENCY IN RELATION TO CHEMICAL CONSTITUTION

BY

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(Received July 7, 1947)

INTRODUCTION

The results described in this paper are not by any means complete, nor are they in every respect unequivocal, but the author feels justified in publishing them as they stand if only because, in the present state of our knowledge of anthelminthics, almost any serious contribution to the field is likely to be of value to others engaged upon its many problems The results of biological tests of about 200 synthetic compounds for anthelminthic properties are described, and the author wishes to express his thanks and profound indebtedness to Prof E Friedmann, to whom the broad plan of the work was due, and without whose expert chemical work the investigation could not have been undertaken With a few exceptions, all the substances tested were prepared in this laboratory by Prof Friedmann, latterly with the assistance of Mrs B Berrill

The aim of the investigation was to discover as much as possible about the relationship between chemical constitution and anthelminthic potency and to find, if possible, new drugs that might be of value in the treatment of nematode infestations. It must be emphasized that, while the term "anthelminthic" as ordinarily employed refers indiscriminately to drugs acting upon parasitic worms of any kind, in the present context it relates specifically to compounds acting upon nematodes

Recent work on the chemotherapy of microbial diseases has led to the discovery of the sulphonamides, penicillin and a number of other specific antibiotics such as atebrin and plasmoquine, and to the beginnings of a rational approach to chemotherapeutics. In the field of anthelminthics, however, although a considerable amount of work has been done, no comparable new drugs have been discovered, and advances can at present only be expected from the classical method of trial and error. While the comparatively recent introduction of hexylresorcinol in cases of nematode infestation undoubtedly represents a great advance

in therapy, especially in ankylostomiasis, the drug most widely used in human ascariasis is still santonin, the active principle of Artemisia maritima, var anthelnunthicum, a remedy that has come down to us from antiquity

CHOICE OF A METHOD

A perusal of the literature indicates that the highest orders of anthelminthic activity may be expected among lactones, some of which have been reported (eg, by Gluschke, 1932, Rosenmund and Schapiro, 1934) to be even more potent than santonin, itself a lactone Our own results, however, indicate that the conclusions of earlier workers in the field must be accepted with considerable reserve, for, although we have tested more than 30 lactones of various types, not one has proved to have activity comparable with or even approaching that of santonin results stand in marked contrast to those of other investigators we attribute to the use of different methods of in vitro testing Relatively few tests appear to have been made on nematode-infested hosts, and many of the observations and conclusions reported in the literature have undoubtedly been prejudiced by the use of annelid material for the detection of anthelminthic potency in vitro This method became current after its acceptance by Trendelenberg (1916), who, finding Ascaris a very refractory material, resorted to the earthworm as a convenient alternative "Dass die Regenwurmer in derselben Weise wie die Spülwürmer auf Santonin mit Erregung reagieren würden schein hochst wahrscheinlich," he wrote Lamson and Ward (1936) condemned the use of earthworms as "irrational" and showed that "a comparative study of the lethality of 121 widely diversified chemical substances on both earthworms and pig Ascaris shows no correlation of action"

The extensive studies of Lamson et al (1935, 1936), which led to the discovery of the valuable

anthelminthic properties of hexylresorcinol, were carried out on intact specimens of pig Ascaris, a notable advance in the technique of in vitro test-But whole Ascaris is not a reliable test As long ago as 1885, von Schroeder studied the influence of various substances upon intact roundworms and was astonished by their resistance to drugs of many kinds. Most surprising of all was his observation that santonin, in spite of its established reputation as an anthelminthic, seemed to have little effect, even when applied in saturated solution over periods of many hours Trendelenberg (1916) referred to a number of similar observations Lamson and Brown (1936) likewise were unable to demonstrate that santonin has any significant effect upon intact Ascaris, and it was only when its action was tested upon isolated anterior fragments of the worm (Rebello and Rico, 1926, Baldwin, 1943a) that any effect of santonin was conclusively demonstrated in vitro Baldwin came to the conclusion that, in fact, santonin acts by paralysing the central nervous system of the parasite without much affecting the peripheral neuro-muscular systems, so that the worms are, in effect, incoordinated by its action, though not "dead" or even moribund to casual observation

Baldwin (1943a) used small, tied-off neuromuscular preparations of pig Ascaris with an intact cuticular laver Unlike earthworm and leech preparations, these do not react to any of a large number of compounds known to be devoid of anthelminthic properties though possessing powerful physiological or pharmacological activity of other kinds, but respond nevertheless to most drugs of acknowledged anthelminthic potency Earthworms and leeches are notoriously sensitive to a wide range of chemical compounds, many of which possess no known anthelminthic activity whatever, and we cannot believe that results obtained with these materials can necessarily be applied without reservation to nematodes

As was pointed out in an earlier paper (Baldwin, 1943a), our normal test preparations of Ascaris fail to respond to acetylcholine, with or without eserine, even at concentrations as high as 1 5,000 Following up this interesting phenomenon we later devised an Ascaris preparation of which the muscle can be directly exposed to the action of any desired drug (Baldwin and Moyle, 1947) in our normal test preparations the musculature is surrounded by an intact cuticular layer. With the new "exposed" preparations we found that the isolated muscle is stimulated by acetylcholine at concentrations of the order of 1 10³-1 10⁶

The response is not demonstrably potentiated by eserine and is, apparently, a pure nicotine action (Baldwin and Moyle, 1948) Earthworm or leech muscle, by contrast, reacts both to the nicotine and muscarine effects of acetylcholine, as well as to adrenaline and a number of other compounds to which isolated Ascaris muscle shows no response whatever To the evidence already adduced by Lamson and Ward (1936) we can thus add direct pharmacological evidence of the unsuitability of annelid material as a test object in anthelminthic studies

The failure of our normal preparations to respond to acetylcholine shows, not that the muscle is insensitive to this compound, but rather that the cuticle is impermeable to it. There is other evidence to show that the cuticle of Ascaris displays highly selective permeability (Trim, 1944) appear, therefore, that a satisfactory anthelminthic must possess at least two attributes (a) be capable of penetrating the nematode cuticle and (b), having so penetrated, have a deleterious action upon nematode tissues Any sound in vitro method for the detection and measurement of anthelminthic activity should therefore be based upon material of nematode origin and with an intact cuticular layer Earthworm and leech preparations of the kind usually employed possess nothing analogous to the cuticular barrier present in our Ascaris preparations, nor is there any guarantee, nor even any a priori probability, that their responses to a given drug can furnish any clue to the action of that drug upon the tissues of the nematode Not only do they belong to an entirely different animal phylum, but the Nematoda as a whole display many unique morphological and physiological features (see, for example, Lapage, 1937)

The use of these Ascaris preparations has certain rather sharply defined limitations, however First, some drugs that fail to gain access to the tissues by way of the cuticle might be able to penetrate by way of the mouth and the alimentary tract, which is occluded in our preparation's Further, our preparations cannot detect directly the anthelminthic potentialities of any compound which, like certain arsenicals (da Costa, 1931), relies upon the tissues or digestive secretions of the host for Last, and most important, our their evocation preparations are unlikely to detect anthelminthic activity in compounds that act otherwise than upon the neuro-muscular apparatus, but the sharp positive correlation between acknowledged anthelminthic potency and positive in vitro responses observed in our earlier experiments (Baldwin,

1943a) would seem to prove that practically all the well-tried, acknowledged anthelminthics do, in fact, act upon the neuro-muscular apparatus Nevertheless, two important drugs failed to evoke any response-viz, phenothiazine and gentian violet, both of which enjoy a high reputation in therapeutics The mode of action of these compounds is still somewhat obscure. It is possible that they undergo conversion within the host into potent anthelminthic products, but it is possible also that they act otherw se than upon the neuromuscular mechanisms, possibly upon the reproductive organs It accordingly follows that our present experiments do not cover the complete field of actual or potential anthelminthics, but in the absence of convenient routine methods for investigating substances that act through other channels the procedure used in the present study seems, in spite of the limitations already enumerated, to offer the most profitable line of immediate approach

Essentially, therefore, our observations cover the activity only of compounds that act upon the neuromuscular apparatus and gain access thereto by way of the cuticle

PROCEDURE

Different anthelminthics undoubtedly act in Lamson and Ward (1932) have different ways divided anthelminthics into five groups according to whether they (1) cause temporary narcosis or paralysis followed by recovery, (ii) narcosis or paralysis followed by death, (iii) injury to the cuticle, (iv) digestion of the parasites, or (v) unexplained death All the drugs in general use have one property in common when applied to our all lead to paralysis of the test preparations anterior region of Ascaris This paralysis is sometimes followed by other phenomena for example by contracture with phenolic drugs such as hexylresorcinol, thymol, and β -naphthol, occasionally the sequelae are strongly characteristic of individual drugs We have not been able to rule out the possibility that the paralysis observed in our tests might sometimes be followed by spontaneous recovery, but no such recovery has been observed in the course of the work Since, therefore, our experiments were mainly of an exploratory nature, we decided to use the common feature of paralysis This is certainly convenient and as our criterion for the most part probably reliable, its use is, we felt, preferable to setting up different criteria for individual drugs or groups of drugs, none of which might be quite comparable with another in any case

All the biological tests reported here were carried out on "anterior preparations" by the method already described (Baldwin, 1943a). The drugs were prepared for testing by the methods described in the same paper, and each compound was tested at several different concentrations, each test usually lasting for 30 min. This enabled us to obtain approximate quantitative data descriptive of the potencies of the compounds by awarding "marks" according to the following scale.

In order to obtain more precise data it would have been necessary to work statistically, and the time taken to do so would more than have outweighed the advantages gained by using this relatively rapid in vitro technique. The more active and therefore more interesting compounds were tested several times and the results averaged, but two or three tests were carried out with every compound. As a precaution against unwilful selection of evidence, each substance was allotted a code number and tested before its identity was disclosed

As standards of reference we carried out numerous experiments with a number of well-known drugs, the results of which are set forth in Table I in terms of the concentration of each required to produce complete paralysis of the anterior region in 20–30 min.

TABLE I
ACTIVITIES OF SOME STANDARD COMPOUNDS

Compound	Concentration producing paralysis in 20–30 min	Nature of prepara tion
Santonin Hexylresorcinol p-Benzylphenyl carbamate Thymol β-Naphthol Oil of Chenopodium Carbon tetrachloride Tetrachloroethylene Chlorbutol	1 100,000 1 10,000 1 5,000 1 5,000 1 5,000 1 5,000 1 2,000 1 2,000 1 1,000	Solution ,, Emulsion ,, Solution

THE ACTION OF SANTONIN

Santonin has been used in the treatment of certain nematode infestations since the dawn of history and its efficacy in practice has never been seriously questioned. But to this day we do not

know what group or radical is responsible for its outstanding activity Trendelenberg (1916) discovered that previously denervated fragments of earthworm muscle remain practically motionless in Ringer's solution but that, on the addition of santonin, their tone rises sharply, leading to the onset of powerful rhythmic contractions which persist as long as santonin is present in the medium The same effect, which is freely reversible, was also evoked by desmotroposantonin, by santonin oxime and by tetrahydrosantonin, all of which contain unmodified the lactone ring present ın santonın itself Santoninic acid, in which the lactone ring is opened, was quite mert, even in relatively concentrated solutions, and Trendelenberg therefore concluded that santonin owes its stimulant action to the presence of its lactone In support of this conclusion he pointed out that other lactones—eg, pilocarpine and coumarine, similarly lose their characteristic effects if the lactone ring is opened

Oswald (1924) pointed out that the physiological activity of many substances is destroyed by the introduction of a carboxyl group into the molecule and suggested that the inactivity of santoninic acid might be due to the presence of its free carboxyl radical rather than to the absence of the lactone ring In support of Trendelenberg's view, Josephson (1931) found that santoninic amide, like the acid, is inert On the other hand, Oshika (1921) found that the ethyl esters of santoning and santonic acids were both active towards earthworm muscle, the corresponding free acids being mert It would therefore appear that, at least as far as earthworm muscle is concerned, the activity of santonin cannot be due solely to the lactone ring

Caius and Mhaskar (1923), working on patients infested with Ascaris administered a number of santonin derivatives and determined the percentages of cases cured by one test treatment. Some of their results are recorded in Table II. The behaviour of santoninic acid might, of course, be due to the ease with which, under acid conditions

TABLE II (Results of Caius and Mhaskar, 1923)

Compound	% patients cured by one test treatment
Santonin Santoninic acid Santonic acid Santonous acid Desmotroposantonin Santonone	80 73 84 67

such as prevail in the stomach, the free acid revers to the lactone

But lactonization is not possible in either santonic (unless perhaps after previous reduction) or in santonous acid, both of which were active Caius and Mhaskar came therefore to the conclusion that the active centre of the santonin molecule is the ketonic group of the unsaturated ring. This is present in santonin, santoninic acid and santonic acid. Santonous acid is usually figured in its enolic form, but that it can undergo ketonization is evident since it forms an oxime with hydroxyl amine and a hydrazone with phenylhydrazine. Desmotroposantonin, which forms neither an oxime nor a hydrazone, does not ketonize and is inactive, santonone, also inert, likewise possesses no ketonic grouping.

Two further possibilities come to mind Anthelminthic activity might be attributable to the presence of an unsaturated ring Lamson et al (1935), for example, found that 4-phenylphenol was very much more active than 4-cyclohexylphenol, Lautenschlager (1921) and Rosenmund and Schapiro (1934) similarly found great\(\text{increases}\) in physiological activity among lactones when phenyl groups were introduced, and we too have found that the introduction of a phenyl radical leads to great increases in anthelminthic activity in several groups of substances, notably among lactones, thiazoles, and pyridines But tetrahydrosantonin (272), although fully saturated, proved in our tests to be as active as santonin itself

A further possibility, that the angular methyl group present in santonin and all its active derivatives may be involved, seems worth investigating in view of the importance of groups of this kind in determining the action of sex hormones. The masculinizing hormones (androsterone, testosterone) possess two angular methyl radicals and the oestrogenic hormones (oestrone, oestradiol) one only, while progesterone, which suppresses some of the characteristic features of feminine

TABLE III

Active as santonin	Inactive
Santonin (31) *B Santonin (32) Santoninic acid (225) Tetrahydrosantonin (272) Santonin oxime (239)	d Desmotroposantonin (223) I Desmotroposantonin (224) I Desmotropo β-santonin) (263) d Santonous acid (261) I Santonous acid (241) Santonic acid (226) Ethyl santonate (256) Allantolactone (34) ψ Santonin (33)

^{*} Isomeric with santonin (Clemo 1934) activity ca 20% that of santonin (Baldwin 1943b)

sexuality while emphasizing others, resembles the androgens in containing two such groups

We have not attempted an exhaustive survey of the structure-activity relationships of the santonin group, but the results listed in Table III, which summarize the results of our experiments on these substances, are of some interest. In all the active compounds with the exception of santoninic acid, which probably owes its activity to the ease with which it reverts to santonin in aqueous solution, we find three structural features in common. These are

- (a) an intact γ -lactone ring,
- (b) a double bond at position 7, and
- (c) an angular methyl group at position 10

The numbering, which is arbitrary, refers to the following structure

One or more of these characters is absent from all the inert derivatives. The inactivity of santonic acid is evidently not due simply to the fact that it contains a free carboxyl radical, for its ethyl ester also is inert, probably, therefore, the inactivity of the acids cannot be attributed solely to their free carboxyl groups

Although there is insufficient evidence to prove that the methyl group at position 10 is essential, it seems very probable indeed that the other two features must both be present if the substance concerned is to possess anthelminthic action may therefore suspect that santonin owes its efficiency to the simultaneous presence of both these features rather than to the presence of either alone One of the difficulties that stand in the way of the further pursuit of the santonin problem is the considerable doubt that still attaches to the structure of some members of the group We may tentatively suggest however that both the lactonic and the ketonic groups contribute to the total anthelminthic potency of the santonin molecule and of its active derivatives. As we shall show in later sections of this paper, both these groupings possess potentialities for anthelminthic activity, and the outstanding potency of santonin itself may perhaps be due to the unique manner in which these two active centres are linked together Finally, though at present there is insufficient evidence to show that the angular methyl group

plays any part in determining the activity of members of this group of compounds, this is a possibility that cannot at present be eliminated and would probably repay further investigation

Clearly, the santonin problem is still a long way from its solution and much more work is needed before any final conclusions can be drawn

Taking santonin as our starting point it seemed desirable to discover whether its powerful activity is shared by other unsaturated ketones on the one hand, or by lactones on the other. Our experiments on these groups of compounds are presented in the next two sections, the remaining sections are devoted to certain other groups—viz, phenols, thiazoles, pyridines, and miscellaneous substances

RESULTS AND DISCUSSION

In the Tables (IV-XXIII) recording the experimental results the name or formula of each compound is followed by the "mark" awarded, the concentration tested and the code number, thus $C_6H_5CO\ CH_3 + + 11,000\ 105$

1 Aliphatic-aromatic ketones

Benzylidene acetone can be derived (on paper) by partial "dissection" of santonin

This compound and a group of related ketones (Table IV, A) proved to possess appreciable anthelminthic potency and attempts were made to increase this by chemical manipulation of the molecules. Activity here seems to be mainly associated with the ketonic group, the presence of one or more unsaturated linkages in the sidechain increasing the activity somewhat Replacement of the phenyl by a furfuryl radical (86) reduced activity considerably

Notable increases in activity were obtained by the introduction of alkyloxy radicals into position 4 of benzylidene acetone but not of acetophenone (Table IV, B) This suggests that the unsaturated side-chain of benzylidene acetone carries greater anthelminthic potentialities than the saturated side-chain of acetophenone Among homologous alkyloxy derivatives maximal activity was found in the 4-ethoxy compound, a fall in potency occurring when the length of this radical was further increased. The influence of the position

~	RI	_	137

	TABLE IV			
A	C ₆ H ₅ CO CH ₃ C ₆ H ₅ CH ₂ CO CH ₃ C ₆ H ₅ CH CH CO CH ₃ C ₆ H ₅ CH CH CH CO CH ₃ C ₆ H ₅ CH CH CH CO CH ₃ C ₆ H ₃ O CH CH CO CH ₃	++ ++ ++ (+)	1 1000 1 1000 1 1000 1 1000 1 1000	105 80 92 120 86
В	C ₀ H ₀ CO CH ₃ 4-CH ₃ O C ₀ H ₄ CO CH ₃ 4-C ₂ H ₅ O C ₆ H ₄ CO CH ₃	++ ++ ++	1 1000 1 1000 1 1000	105 95 93
	C ₆ H ₃ CH CH CO CH ₃ 4-CH ₃ O C ₆ H ₄ CH CH CO CH ₃ 4-CH ₃ CH ₂ O C ₆ H ₄ CH CH CO CH ₃ 4-CH ₃ CH ₂ O C ₆ H ₄ CH CH CO CH ₃ 4-CH ₃ CH ₂ O C ₆ H ₄ CH CH CO CH ₃ 4-(CH ₃) ₂ CHO C ₆ H ₄ CH CH CO CH ₃ 4-CH ₃ CH CH ₂ O C ₆ H ₄ CH CH CO CH ₃ 4-CH ₃ CH ₂ CH ₂ O C ₆ H ₄ CH CH CO CH ₃	++ ++ ++ (+) ± + +	1 1000 1 2000 1 5000 1 5000 1 2000 1 2000 1 2000	92 113 81 82 89 84 83
С	4-CH ₃ O C ₆ H ₄ CH CH CO CH ₃ 3-CH ₃ O C ₆ H ₄ CH CH CO CH ₃ 2-CH ₃ O C ₆ H ₄ CH CH CO CH ₃	++ + (+)	1 2000 1 2000 1 2000	113 100 98
	4-C ₂ H ₆ O C ₆ H ₄ CH CH CO CH ₃ 3-C ₂ H ₆ O C ₆ H ₄ CH CH CO CH ₃ 2-C ₂ H ₅ O C ₆ H ₄ CH CH CO CH ₃	+ + + (+)	1 5000 1 2000 1 2000	81 99 97

of the alkyloxy group was next determined, maximal and minimal activities being found for positions 4 and 2 respectively (Table IV, C) The effects of pairs of alkyloxy radicals are not additive in the acetophenone series, and in the benzylidene series are actually antagonistic (Table

V, A) The introduction of allyl groups served only to diminish the existing activity (Table V, B)

The hitherto unsubstituted methyl group of the ketonic side-chain was now modified by the introduction of further CH_3 or C_2H_5 radicals This

TABLE V

A Acetophenone d	erivatives			4	\sum_{coc}	H _s	•
4-ethoxy 2 4-diethoxy				++		1 2000 1 2000	93 218
Benzylidene acetone derivatives			4	² >Сн (СН СО СН,		
4-methoxy- 4-ethoxy- 3 4-dimethoxy- 3 4-methylenedioxy- 3-methoxy-4-ethoxy 2-methoxy-4-ethoxy	-			++ + - (+) ± (+))	1 2000 1 5000 1 2000 1 2000 1 2000 1 2000	113 81 91 85 88 104
В	Benzylidene acetone	derivative	es			Same compo without all	
2-methoxy-4-allyl- 2-ethoxy-4-allyl 4-ethoxy-3-allyl 2-methoxy-4-ethoxy- 3-methoxy-4-ethoxy-		± ± -	1 2000 1 2000 1 2000 1 2000 1 2000	109 110 106 108 107	(+) (+) + - (+) ±	1 2000 1 2000 1 5000 1 2000 1 2000	98 97 81 104 88

TABLE VI

4-CH₃O C₀H₄CH₂CH₂ CO CH₃	(+)	1 2000	121
4-CH₃O C₀H₄CH₂CH₂ CO CH₂CH₃	+	1 2000	122
4-CH₃O C₀H₄CH₂CH₂ CO CH₂CH₂CH₃	(+)	1 2000	123
4-CH ₃ O C ₆ H ₄ CH CH CO CH ₃	++	1 2000	113
4-CH ₃ O C ₆ H ₄ CH CH CO CH ₂ CH ₃	++	1 2000	115
4-CH ₃ O C ₆ H ₄ CH CH CO CH ₂ CH ₂ CH ₃	(+)	1 2000	117
4-C ₂ H ₆ O C ₆ H ₄ CH CH CO CH ₃	+	1 5000	81
4-C ₂ H ₆ O C ₆ H ₄ CH CH CO CH ₂ CH ₃	+	1 5000	112
4-C ₂ H ₆ O C ₆ H ₄ CH. CH CO CH ₂ CH ₂ CH ₃	1nsol	uble	111
4-Cl C₅H₄CH CH CO CH₃	+++	1 2000	101
4-Cl C₅H₄CH CH CO CH₂CH₃	insol	uble	102
C¹H³O CH CH CO CH³CH³	(+)	1 1000	86
	++	1 2000	114

Т	Ά	B	LE	V	Π

A Cyclohexadiones	R ₁ CH——CH ₂ R ₂ CH CO CO——CH ₂			
R_{i}	R ₂			
C ₈ H ₅ C ₈ H ₅ C ₆ H ₅ 4-CH ₃ O C ₆ H ₄	H CN CO ₂ H H	_ _ _ _	1 1000 1 1000 1 1000 1 1000	118 124 103 126
B α-Ketonic acids		1 .		1
	C ₆ H ₅ CH CH CO COOH 4-CH ₃ O C ₆ H ₄ CH CH CO COOH C ₆ H ₅ CH CH CH CH CO COOH C ₄ H ₃ OCH CH CO COOH	 	1 1000 1 1000 1 1000 1 1000	96 125 116 87

was done with derivatives of anisylacetone, 4-methoxybenzylidene acetone, 4-ethoxybenzylidene acetone, 4-ethoxybenzylidene acetone, and furfurylidene acetone. The results are shown in Table VI. There is a slight but probably significant increase of activity with the addition of one $-CH_2$ — unit, followed by a decline when a second such group is introduced

Since cyclization of the side-chain of physiologically active substances sometimes leads to important increases in their potency, and we had so far failed to obtain activities greater than about + at 1 5,000, a series of cyclic diketones was tested these may, for our purposes, be regarded as derived from cinnamylidene acetone (120) These diones proved to be completely inert and so too did a series of α -keto-acids corresponding to some of the parent ketones (Table VII)

The first halogenated products tested seemed to offer a prospect of greater activities, but no further increases could be obtained by the further introduction of alkyloxy radicals (Table VIII) influence of phenolic (OH) groupings was next studied Numerous phenols are known to possess important anthelminthic properties and some of these, notably thymol, β -naphthol, and hexylresorcinol have found extensive employment in clinical medicine and veterinary science The first attempts in this direction were somewhat discouraging, for the introduction of (OH) at position 4 in acetophenone, and at position 2 in benzylidene acetone, completely destroyed such activity as was formerly present (Table IX) Further work, however, brought to light some interesting phenomena Whereas the introduction of (OH) at position 4 in acetophenone resulted in inactivation, substitution

-TABLE VIII

C ₆ H ₅ CO CH ₃ C ₆ H ₅ CO CH ₂ Br 4-CH ₃ O C ₅ H ₄ CO CH ₃ 4-CH ₃ O C ₆ H ₄ CO CH ₂ CI	{++ {+++ ++ ++	1 1000 1 2000 1 5000 1 1000 1 1000	105 171 95 181
C₀H₀CH CH CO CH₃ 4-Cl C₀H₄CH CH CO CH₃	++ +++	1 1000 1 2000	92 101
TABLE IX			
C ₆ H ₅ CO CH ₃ 4-HO C ₆ H ₄ CO CH ₃ 2-HO C ₆ H ₄ CO CH ₃ 2 4-(HO) ₂ C ₆ H ₃ CO CH ₃ 2-C ₆ H ₆ O C ₆ H ₄ CO CH ₃ 4-C ₂ H ₆ O C ₆ H ₄ CO CH ₃ 2 4-(C ₂ H ₈ O) ₂ C ₆ H ₃ CO CH ₃ 2-C ₆ H ₆ O C ₆ H ₃ CO CH ₃ 2-C ₆ H ₆ O C ₆ H ₃ CO CH ₃ 2-HO C ₆ H ₃ CO CH ₃ 2-HO C ₆ H ₃ CO CH ₃	++ -++ {+++ (+) ++ + {+++ (+)	1 1000 1 1000 1 2000 1 2000 1 2000 1 1000 1 2000 1 1000 1 2000 ca 1 2000 1 1000 1 2000	105 94 213 127 228 93 218 217
C ₆ H ₆ CH CH CO CH, 2-HO C ₆ H ₄ CH CH CO CH, 2-CH ₅ O C ₆ H ₄ CH CH CO CH, 2-C ₂ H ₆ O C ₆ H ₄ CH CH CO CH,	++ - (+) +	1 1000 1 1000 1 2000 1 2000	92 119 98 97

at position 2 approximately doubled the activity, while simultaneous hydroxylation at positions 4 and 2 yielded an inert product. But the introduction of (OH) at position 2 in 4-ethoxyacetophenone had little influence, nor was there much change when, instead of (OH), a second ethoxy radical was placed in position 2 Further evidence of the mactivating influence of the 4-hydroxy group was obtained with 2-ethoxy-4-hydroxyaceto-Thus a phenolic (OH) abolishes phenone (217) the activity of acetophenone when placed in the 4-position but tends to increase it when placed in position 2, unless the latent potentialities of the substance have already been evoked by the substitution of an alkyloxy radical in position 4, when the effects of the two substituents are not additive Essentially the same phenomena were observed in a series of hydroxylated derivatives of halogenated acetophenones (Table X) Benzylidene acetone, unlike acetophenone, is inactivated by hydroxylation at position 2

Thus no combination of the potentiating radicals used in these experiments raised the activity of the compounds beyond a value of about + at 1 5,000 When, by the introduction of one potentiating grouping a relatively high order of potency had been developed, the addition of a

TABLE X

C ₆ H ₆ CO CH ₃ C ₄ H ₆ CO CH ₂ Br 4-HO C ₆ H ₄ CO CH ₂ Cl 2-HO C ₅ H ₄ CO CH ₂ Cl 2 4-(HO) ₂ C ₆ H ₃ CO CH ₂ Cl 3 4-(HO) ₂ C ₆ H ₃ CO CH ₂ Cl 4-CH ₃ O C ₆ H ₄ CO CH ₂ Cl	++ {+++ + + {++ 	1 1000 1 2000 \ 1 5000 \ 1 1000 1 5000 1 1000 1 1000 \ 1 2000 \	105 171 183 200 170 168 181
2-HO 4-C ₂ H ₅ O C ₆ H ₃ CO CH ₂ Cl	+	1 2000	203

T 4	TOT	\mathbf{r}	vi
14	. KI	·r	

	TABLE AT				
2 4-(HO) ₂ C ₆ H ₃ CO CH ₃ 2 4-(HO) ₂ C ₆ H ₃ CO CH ₂ CH ₃ 2 4-(HO) ₂ C ₆ H ₃ CO CH ₂ CH ₂ CH ₃ 2 4-(HO) ₂ C ₆ H ₃ CO CH ₂ CH ₂ CH ₂ CH ₃ 2 4-(HO) ₂ C ₆ H ₃ CO CH ₂ CH ₂ CH ₂ CH ₂ CH ₃ 2 4-(HO) ₂ C ₆ H ₃ CO CH ₂ CH ₂ CH ₂ CH ₂ CH ₂ CH ₃	+ + +++ (+)	1 2000 1 2000 1 2000 1 10,000 1 10,000	 ++ +++ (+) ++ +	1 2000 1 1000 1 1000 1 5000 1 5000 1 5000	127 229 230 237 240 242
2 4-(HO) ₂ C ₆ H ₃ CH ₂ CH ₂ CH ₃ CH ₂ CH ₂ CH ₂ CH ₂ CH ₃ CH ₂ CH ₂ CH ₂ CH ₃ CH ₂ CH ₃ CH ₂ CH ₂ CH ₂ CH ₂ CH ₃ CH ₃ CH ₂ CH ₂ CH ₂ CH ₂ CH ₃			+ + + +	1 2000 1 10,000 1 10,000	259 5 258
	TABLE XII				
C ₈ H ₆ C 4-HO C ₈ H ₄ C 4-C ₂ H ₆ O C ₈ H ₄ C	$O C_8H_5$ $O C_6H_5$ $O C_6H_5$	(+) ++ (+)	1 1	1000 1000 2000	220 132 133
2-HO C₀H₄C 2-C₂H₅O C₀H₄C	$O C_6H_5$ $O C_6H_5$		ca 1	1000 1000	219 227

second potentiating radical was liable to diminish rather than increase it

A series of alkylated resorcinyl ketones was now examined Resorcinyl methyl ketone itself was mert, but it was thought that the introduction of longer alkyl radicals into the side-chain might reveal some latent anthelminthic potency, much as the bacterial (Leonard, 1924) and the anthelminthic (Lamson, Brown, and Ward, 1935) potentialities of resorcinol are augmented The results (Table XI) show that marked anthelminthic properties appear in resorcinyl ethyl ketone (229), increase in intensity as the alkyl chain is lengthened, and reach a maximum in the valeryl ketone (240) Further lengthening of the chain is attended by diminishing activity These results run roughly parallel to those obtained by Lamson, Brown, and Ward (1935) for the alkyl resorcinols, but the ketones show appreciably less activity than the corresponding non-ketonic alkylresorcinols (Table XI)

Finally, a group of substances derived from benzophenone and containing two aromatic rings was investigated. The results (Table XII) contain little of interest beyond showing that the effects of hydroxylation at positions 2 and 4 are precisely opposite to those observed in the acetophenone series (Table IX). The effect of the ethoxy radical, as in benzylidene acetone, is greater in position 4 than in 2

The conclusions reached regarding the behaviour of these ketones may be summarized as follows (1) The ketonic group of aliphatic-aromatic ketones carries potentialities for anthelminthic activity which approach nearly to those of thymol and

 β -naphthol (11) This latent potency can be evoked by substitution of alkyloxy or phenolic radicals, in the benzene ring, or by halogenation (111) The influence of these potentiating radicals varies from one group of ketones to another and with the position of substitution Finally (1v), the effects of these potentiating radicals are not additive and may, in fact, be antagonistic in certain compounds

2 Lactones

Since Trendelenberg (1916) came to the conclusion that the anthelminthic properties of santonin are due to its lactone ring, many new lactones have been prepared and tested, for example by Lautenschlager (1921), von Oettingen (1929), Gluschke (1932), and Rosenmund and Schapiro (1934) Activity greater than that of santonin has been claimed for some of these products

Lautenschläger (1921) tested a series of γ -lactones (γ -butyro-lactone, γ -valerolactone, paraconic acid lactone and a number of sugar lactones and betaines) The simpler compounds had little action upon earthworm muscle, intact earthworms or the cardiac muscle of the frog, but great increases in activity with respect to these materials were obtained by the introduction of phenyl radicals. Phenyl butyrolactone and phenyl paraconic acid lactone were about half as active as santonin upon earthworm preparations, while a third product, phthalide (α β -benzbutyrolactone), was as active as santonin itself. A considerable number of related compounds also showed a high order of activity

von Oettingen (1929) tested butyrolactone, valerolactone, valerolactone carboxylic acid, isocaprolactone, α - and β -angelica lactones and the dilactone of acetone di-acetic acid, all of which had a more or less depressant action upon isolated earthworm muscle This contrasts sharply with the powerful stimulant action of santonin upon the same tissue (Trendelenberg, 1916) Oettingen found that activity was greatly increased by the introduction of methyl or carboxyl groups into the lactone ring or by the introduction of a double bond At concentrations of $0.04 M_{\odot}$ β -angelica lactone, valerolactone carboxylic acid and the dilactone were as active as santonin, though less so in more dilute solutions Oettingen and Garcia (1929) then showed that β -angelica lactone removed all the roundworms from 7 out of 10 infested cats—one of the few published experiments in which lactones other than santonin have been tested in infested hosts

Gluschke (1932) prepared a number of lactones and claimed that certain lactones derived from α -tetralone, and nearly related to santonin itself, equalled or surpassed the latter in activity—viz, syntonins a and b (I and II) In these experiments again the test object consisted of earthworm muscle

Rosenmund and Schapiro (1934), following up the work of Lautenschlager (1921), prepared a series of substituted y-butyrolactones and tested their activity upon leech muscle and intact specimens of Ascaris They state that the o-cresol ether and anisole derivatives of y-butyrolactone were from 3 to 4 times more active than santonin and that there was, moreover, a close parallel between the responses of leech muscle and of the intact roundworms Yet, as has been pointed out, santonin itself has little or no evident action upon intact Ascaris (von Schroeder, 1885, Lamson et al. 1935, 1936) It is accordingly difficult to assess the validity of Rosenmund and Schapiro's observations and conclusions

The variety of lactones which might have been made and tested is so large that we felt it necessary to restrict the scope of our work to some extent We have however tested several groups of lactones in which a high order of activity was to be anticipated from the results of our predecessors in this field, together with a number of miscellaneous

lactones representing a considerable variety of chemical types

a-Angelica lactone (231) and its anisal derivative (234), the dilactone of acetone diacetic acid (238). and copper glycine (236), which has interesting structural resemblances to the dilactone, were tested at a concentration of 1 1,000 a-angelica lactone showed any activity (+ at 1 1,000) although, in view of the claims of von Oettingen (1929) and Rosenmund and Schapiro (1934), the anisal compound if no other might have been expected to show a very high order of activity More nearly related to santonin were the d- and l-desmotroposantonins (223, 224, III) and l-desmotropo- β -santonin (263) These com pounds are very insoluble and were tested in saturated solutions Had they possessed activity in any way comparable with that of santonin it would, according to our estimates of their solubilities, have been detectable in our experiments, but uniformly negative results were obtained Of

particular interest in relation to Gluschke's (1932) claims was an observation that alantolactone (34, IV), a substance even more closely allied to santonin than are the syntonins, was totally mert when tested at 1 2,000. According to von Oettingen (1929) the presence of a double bond augments the activity of the lactone ring so that a particularly high order of potency might have been expected here

A series of derivatives of γ -butyrolactone was also examined and here it was found that the activity of γ -phenylbutyrolactone itself approaches that of santonin in fairly high concentrations. But the two substances are in no way comparable at lower concentrations, phenylbutyrolactone giving dubious or slight activity at 1 10,000 whereas santonin is still powerfully active at 1 100,000 Alkyloxylation removed the activity. Our results with this series of compounds (Table XIII) are entirely at variance with those of Rosenmund and Schapiro (1934)

In confirmation of an earlier observation (Baldwin, 1943a) coumarine (V) was found to be active and umbelliferone (7-hydroxycoumarine) inert. 3-Hydroxycoumarine and 7-ethoxycoumarine were also tested Chromone (VI), 2-cou-

TABLE XIII

Phenylbutyrolactone 4-Ethoxyphenylbutyrolactone 2-Methyl-4-ethoxyphenylbutyrolactone 3-Methyl-4-ethoxyphenylbutyrolactone 2-Ethoxy-5-methylphenylbutyrolactone 2-Naphthylbutyrolactone	+++ ± ± ± -	1 5000 1 1000 1 1000 saturated 1 1000 saturated	260 262 266 267 264/5 268
TABLE XIV			
Coumarine (V) 3-Hydroxycoumarine 7-Hydroxycoumarine 7-Ethoxycoumarine Chromone (VI) Ethyl chromone-2-carboxylate 2-Coumaranone (VII) 3-Coumaranone (VIII) 6-Hydroxy-3-coumaranone 6-Ethoxy-3-coumaranone	+ - - (+) {++ (+) (+) {++ + + - -	1 1000 1 2000 1 1000 1 2000 1 2000 1 1000 1 1000 1 2000 1 2000 1 2000 1 2000	74 211 78 210 245 257 233 186 191 190

maranone (VII), and 3-coumaranone (VIII) were also examined, together with some of their derivatives, in view of their relation to phthalide (IX) which, according to Lautenschlager (1921), is as active as santonin The results obtained with this group of compounds are listed in Table XIV It

is noticeable first of all that in none of these fusedring compounds does there appear any activity approaching that of santonin, or even comparable with that of phenylbutyrolactone (Table XIII) This would appear to indicate that higher potencies are associated with separated than with fused rings, a phenomenon which, as we shall see, appears in other groups of compounds

All four parent compounds (V-VIII) may be regarded as cyclized derivatives of o-phenols, and

all possessed some activity This seems to confirm the observation that hydroxylation of acetophenone in position 2 (Table IX) leads to marked increases of activity The most active member of the group, 3-coumaranone (VIII), may be regarded as a cyclized form of 2-hydroxyacetophenone, with the activity of which $(++^a)$ at 1 2,000) its own is comparable 6-Hydroxy-3-coumaranone, which may be compared with 2 4-dihydroxyacetophenone (- at 1 2,000), was mert, but there was no return of activity when the hydroxyl group at position 6 was replaced by an ethoxy radical (cf 2-hydroxy-4-ethoxyacetophenone, 215) Hydroxylation of coumarine at position 7 similarly led to loss of activity, but in this case replacement of (OH) by an ethoxy radical was attended by the return of some degree of activity

Three phenylated ketolactones derived from butyrolactone were also examined and found to be inert, while clavatin, which is believed to contain a lactone ring (Raistrick, 1943), and the azlactone of resorcinol aldehyde were also inert (Table XV)

Taken as a whole these results show that considerable anthelminthic activity is in some compounds associated with lactonic structure. But the appearance of such properties among lactones is very sporadic indeed, so much so that they may well be purely fortuitous and associated with other structural features. Certainly there is no evidence

TABLE XV

Resorcinol aldehyde azlactone insoluble 201	Ethyl 2-phenyl-4 5-diketotetrahydrofurane-3-carboxylate 2-Phenyl-3-acetyl-4 5-diketotetrahydrofurane 2-Phenyl-3-cinnamyl-4 5-diketotetrahydrofurane Clavatin Resorcinol aldehyde azlactone	 Insolu	1 1000 1 1000 saturated 1 5000 uble	248 255 253 232 201	
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to support the notion of a specific relationship between anthelminthic potency and lactonic structure. One outstanding point is the greater activity of compounds with separated as opposed to fused rings.

3 Phenols

Many phenols are known to possess anthelminthic activity, and the work of Lamson et al (1935, 1936) added many new ones to the list Several phenols and phenolic derivatives have found wide clinical and veterinary employment, notably β -naphthol, thymol, and hexylresorcinol, all of which gave positive results in our tests Our results with a number of phenolic derivatives of the aliphatic-aromatic ketones have already been described (Tables IX-XII)

Attempts were made to find new active derivatives of active phenols and a study was also made of several phenolic families that hitherto have not been systematically investigated. A number of phenolic acetates, chloroacetates, methylsulphonates, benzenesulphonates, p-toluenesulphonates, cinnamates, and carbamates have been prepared and tested, but proved for the most part to be inert or insoluble, apart from the carbamates (Tables XVI, XVII). The carbamates showed

TABLE XVI

Phenyl chloracetate	+	1 2000 月	180
Thymol	+	1 5000	7
Thymyl chloracetate		1 1000	179
Thymyl methylsulphonate		1 1000	243
1-Naphthol 2-Naphthol 2-Ethoxynaphthalene 2-Naphthyl acetate 2-Naphthyl carbamate 2-Naphthyl methylsulphonate	+	1 5000	182
	+	1 5000	6
	+	1 1000	204
	-	1 2000	208
	+	1 5000	221
	n	soluble	247
Ethyl salicylate	(+)	1 1000	206
2-Aminophenol		1 1000	165
2-Aminothiophenol		soluble	166

activity of the same order as the parent phenols and, in view of their lesser toxicity, are likely to be of practical use

Among the lactones previously examined, greater activity had been found among those containing independent than those containing fused ring systems. It seemed desirable, there fore, to discover whether the same rule might also apply among phenois A number of phenylated phenols were accordingly examined with a view to comparing their activity with that of the naphthols One member of this series, 4-benzylphenyl carbamate, is already in use on a fairly large scale under a variety of proprietary names The results are listed in Table XVII where, as in Table XVI, the only useful derivatives were the relationships carbamates Several interesting appeared, however, especially with reference to the position of the phenolic (OH) group

2-Hydroxydiphenyl (135) was highly active (+ at 1 10,000) and the 4-compound insoluble, these effects resemble those observed with aceto phenone (Table IX) and contradict the results of Lamson et al (1935), who found the 4-compound strongly and the 2-derivative only feebly active In the diphenylmethane series these effects were reversed, 4-hydroxybenzylphenol (128)about 3 times as active as the 2-compound (130), here our results are in agreement with those of Lamson et al (1935) In strong contrast to our observations among the ketones we found that, among phenylphenols and benzylphenols alike, the replacement of (OH) by an ethoxy radical, whether in the 2- or the 4-position, always abolished activity

Compared with 1-naphthol (+ at 1 5,000), with its fused rings, 2-hydroxydiphenyl, with independent rings gave a higher order of activity (++ at 1 5,000), thus falling into line with the results found among lactones Further general confirmation is to be found in the fact that among the phenylphenols and benzylphenols alike, the active hydroxy derivatives showed potencies higher than that of 1- and 2-naphthols The most potent substance discovered in this group was 2-hydroxydiphenyl carbamate (136), and it is noteworthy that this compound was found more active than 4_Benzylphenyl phenol $\sim (135)$ parent carbamate, a drug that has done good service in practice, is less active than the phenol from

TABLE XVII

A	2-HO C ₆ H ₄ C ₆ H ₅ 2-C ₂ H ₆ O C ₆ H ₄ C ₆ H ₅ 2-CH ₃ CO O C ₀ H ₄ C ₆ H ₅ 2-H ₂ N CO O C ₆ H ₄ C ₆ H ₅ 2-CH ₃ SO ₂ O C ₆ H ₄ C ₆ H ₅ 2-C ₆ H ₅ SO ₂ O C ₆ H ₄ C ₆ H ₅ 2-(4 ¹ -CH ₃ C ₆ H ₄ SO ₂)O C ₆ H ₄ C ₆ H ₅ 2-C ₆ H ₆ CH CH CO O C ₆ H ₄ C ₆ H ₅ 4-HO C ₆ H ₄ C ₆ H ₅ 4-C ₂ H ₆ O C ₆ H ₄ C ₆ H ₅ 4-C ₂ H ₆ O C ₆ H ₄ C ₆ H ₅ 4-CH ₃ CO O C ₆ H ₄ C ₆ H ₅ 4-H ₂ N CO O C ₆ H ₄ C ₆ H ₅	\begin{array}{c c c c c c c c c c c c c c c c c c c	135 206 207 136 0 244 214 212 188 134 209 205
В	2-HO C ₀ H ₄ CH ₂ C ₆ H ₅ 2-C ₂ H ₅ O C ₆ H ₄ CH ₂ C ₆ H ₅ 4-HO.C ₆ H ₄ CH ₂ C ₆ H ₅ 4-C ₂ H ₅ O C ₆ H ₄ CH ₂ C ₆ H ₅ 4-H ₂ N CO O C ₆ H ₄ CH ₂ C ₆ H ₅ 4-CH ₃ SO ₂ O C ₆ H ₄ CH ₂ C ₆ H ₅	+ 1 5000 - 1 2000 {+++ 1 2000 +++ 1 5000 - 1 2000 ++ 1 1 5000 - 1 1000	131 128 129 129 53

which it is derived, and less active also than 2-hydroxydiphenyl carbamate

The results obtained with the carbamates suggested an investigation of some unsaturated amides and substituted ureas, none of which however yielded results of any great interest or importance (Table XVIII) Tests on the tolyl compounds were limited by the very sparing solubilities of these substances

The principal conclusion reached regarding the phenols may be summarized as follows (1) The already abundant evidence for the anthelminthic

potency of phenols and their carbamates has been generally confirmed (11) The position of the (OH) radical has different effects in different chemical groups (111) Phenols containing independent ring systems are more active than those in which the rings are condensed, and (1v) one compound with considerable promise has been discovered—viz, 2-hydroxydiphenyl carbamate

4 Thiazoles

Recent work on the chemotherapy of bacterial diseases has emphasized the importance of certain

TABLE XVIII

C ₆ H ₆ CH CH CO NH ₂ C ₆ H ₆ CH CH CO NH CH ₃ C ₆ H ₆ CH CH CO N(CH ₃) ₂ C ₆ H ₆ CH CH CO N(CH ₂ CH ₃) ₂ C ₆ H ₆ CH CH CO N(CH ₂ CH ₃) ₂ C ₆ H ₆ CH CH CO NH CH ₂ CH ₂ CH ₃ C ₆ H ₆ CH CH CO NH CH CH ₂ CH ₂ CH ₃	(+) (+) (+) (+)	1 1000 1 2000 1 1000 1 1000 1 1000 1 2000 1 2000	189 195 196 193 194 192 187
C ₆ H ₅ NH CO NH ₂		1 1000	76/284
C ₆ H ₅ NH CS NH ₂		1 1000	285
2-CH ₃ C ₆ H ₄ NH CO NH ₂		1 10,000	282
2-CH ₃ C ₆ H ₄ NH CS NH ₂		1 10,000	280
3-CH ₃ C ₆ H ₄ NH CO NH ₂		1 1000	283
4-CH ₃ C ₆ H ₄ NH CO NH ₂		1 10,000	281
4-CH ₃ C ₆ H ₄ NH CS NH ₂		1 10,000	279
2-Naphthyl urea	ınşol		286
2-Naphthylthiourea	ınsol		287

TABLE XIX

_	~ 1 1000	198
1 -		
1 - 1	1 1000	175
1 ++ !	1 2000	172
_	1 2000	199
-	1 1000	185
ınsol	uble	184
_]	1 1000	167
	1 1000	169
++	1 2000	173
-	1 2000	177
-	1 2000	52
1 1		Į.
	 ++	- 1 2000 - 1 1000 - 1 1000 - 1 1000 - 1 1000 ++ 1 2000 - 1 2000

ring compounds, notably thiazoles and pyridines, as potential antibiotics. It seemed worth while therefore to see whether derivatives of these compounds might hold out any promise of useful anthelminthic activity. Among the thiazoles, 2-aminothiazole provided a convenient starting point,

This compound was inactive, but appreciable activity appeared with the introduction of a phenyl radical to form 2-amino-4-phenylthiazole (Table XIX) Hydroxylation of the benzene ring, whether at position 2 or 4 only served to inactivate the products, while alkyloxylation rendered them insoluble Sulphathiazole was quite inactive

Further tests were made with two benzth azoles, in which the component rings are fused Contrary to our experience with lactones and phenols, ring fusion in the present series had little effect, while the introduction of a second phenyl radical at position 2 of the thiazole ring yielded an inert product Little prospect of useful potency was found among these substances, therefore, and we went on to study a series of pyridine derivatives

5. Pyridines

Pyridine itself already showed an activity of the same order as that of thymol and β -naphthol A number of substituted products (Table XX)-showed no greater activity, with the exception of 2-aminopyridine. A ketonic derivative, 1-methyl-2-pyridone, was inert. Nikethamide, a synthetic derivative of pyridine, was inactive, while arecoline, the active principle of the betel nut, proved to be relatively feeble. With the introduction of a second ring to form 4-benzylpyridine there was a sharp rise in activity, sulphapyridine, however, was inert.

In order to explore further the effects of a second ring, a series of dipyridyls was examined, and here was found the highest order of activity encountered in the course of the work (Table XXI) 2–2'-Dipyridyl showed activity comparable with that of santonin and was far more active than any other of the four dipyridyls prepared This high potency appears to be specifically associated with the 2–2'-linkage, for there was a profound fall of potency when this was shifted

TABLE XX

		I —
{+++ +	1 2000 \ 1 5000 }	143
\ \\ \(\(\) \\ \(\) \\ \(\) \\ \(\)	1 2000 \ 1 5000 \	144
\ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \	1 1000 \ 1 2000 f	142
	1 1000	138
\ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \	1 2000 - 1 5000 1 10 000	139
\ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \	1 5000 \ 1 10.000 f	153
'-	1 1000	59
++	1 1000 1 1000	58 57
	\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \	\begin{array}{c c c c c c c c c c c c c c c c c c c

TABLE XXI

2-2'-Dipyridyl 2-3'-Dipyridyl 3-4'-Dipyridyl 4-4'-Dipyridyl 2-Methyl-4-4'-dipyridyl 4-Pyridylpyridinium chloride 2-2'-2"-Tripyridyl 2-2'-2"'-Tetrapyridyl	{+++ + ± ± - - {++ ± n- insol	1 50,000 \\ 1 100,000 \\ 1 1000 \\ 1 1000 \\ 1 1000 \\ 1 1000 \\ 1 1000 \\ 1 50,000 \\ 1 100,000 \\ uble	137 158 152 147 154 151 145
---	--	--	---

to the 2-3'-position 2-2'-2"-Tripyridyl was rather less active than the dipyridyl at the same concentrations, but the difference is probably attributable to its higher molecular weight. The corresponding tetrapyridyl was insoluble

Following up the clue afforded by 2-2'-dipyridyl a number of other compounds were prepared containing pairs of nitrogen atoms linked to adjacent carbon atoms, but no activity was discovered here until we came to the phenanthrolines (Table XXII) Of these, 4 5-phenanthroline showed activity equal to that of 2-2'-dipyridyl, the other two being feebly active or inert. We are led, therefore, to the conclusion that a particularly high order of anthelminthic potency is associated, perhaps very

which is common to the two most active compounds

It seems possible that, by suitable chemical manipulation, 2–2'-dipyridyl and 4 5-phenanthroline might be made the basis of new and valuable anthelminthics Harwood (1934) has pointed out that the most useful anthelminthic drugs for the treatment of intestinal infestations have melting points below about 80°C and are only sparingly soluble (1 1,000 or less) in water Probably, therefore, these new substances themselves are too soluble to be of much value in the removal of intestinal nematodes but, if their toxicity is not excessive, they might conceivably prove useful in infestations of the blood and lymphatic systems without further modification Conceivably, it might be possible to produce

TABLE XXII

Ethylene diamine hydrate 1 2-Diaminobenzene (o-phenylene diamine) 2 5-Dimethylpyrazine 2 5-Disodium pyrazine dicarboxylate Benzpyrazine (quinoxaline)	- - - -	1 1000 1 1000 1 1000 1 1000	163 165 157 150
- N N CH-CH	_	1 1000	148
4 5-Phenanthroline CH=CH N	++	1 100,000	146
1 5-Phenanthroline CH=CH N	+	1 1000	156
1 8-Phenanthroline CH=CH-N-N-N-N-N-N-N-N-N-N-N-N-N-N-N-N-N-N-N	-	1 1000	149

sparingly soluble derivatives that would be of value in intestinal infestations

6 Miscellaneous antibiotics

In addition to the groups of substances already mentioned, a considerable number of miscellaneous antibiotics were tested in the course of the experiments. These included a number of well-known and important substances, but none proved to show any anthelminthic activity whatever (Table XXIII). These negative results help

TABLE XXIII

	1	1	_
Acetarsone	_	1 500	54
Alepol	_	1 1000	159
Atebrin	_	1 1000	140
Bayer 205	_	1 2000	71
Clavatin	_	1 5000	232
2 7-Diaminoacridine	l —	1 2000	67
Hydnocarpus 01l		1 1000	161
Neosalvarsan	_	1 2000	55
Penicillin	<u> </u>	480 O U /	66
	ĺ	ml	
Sulphaguanidine	-	1 1000	160
Sulphamethazine		1 1000	250
Sulphanilamide	-	1 100	51
Sulphasuxidine	-	1 1000	254
Sulphathiazole		1 2000	52
Tartar emetic		1 1000	164
m 41		1 4 4000	
Tyrothricin	-	ca 1 1000	270

to emphasize the necessity, already stressed in this paper, of choosing as test material a tissue preparation that properly represents the organism it The biological activity of is desired to attack antibiotics seems in general to be specifically limited to particular organisms or groups of closely Among parasitic helminths related organisms this same specificity is well known santonin, which acts upon nematodes, is devoid of activity upon tape-worms, for example, while pelletierine acts upon tape- but not upon round-worms and so At the same time, however, agents are available that attack both types, these include numerous phenols, but these agents are bactericidal as well and are indeed, members of the category general protoplasmic poisons"

SUMMARY

- 1 This paper reports the results of tests carried out *in vitro* on over 200 chemical compounds for the detection of anthelminthic potency. The technique employed has certain limitations which are enumerated and discussed in the text
- 2 Significant activity is found among aliphaticaromatic and aromatic-aromatic ketones, but in spite of numerous structural modifications and

- manipulations nothing approaching the activity of santonin has been discovered in this group
- 3 Among lactones considerable activity was observed, but here again the activity of santonin far exceeds that of any other lactone tested in these experiments. These facts appear to support the suggestion that the efficacy of santonin is due in part to its ketonic and in part- to its lactonic structure, but that its outstanding anthelminthic power is due to the simultaneous presence of both and to the unique manner in which they are combined together, rather than to either alone
- 4 Among the lactones, phenols, and pyridines tested it was observed that anthelminthic activity increased with the addition of a second (usually a benzene) ring to the parent molecule, and that activity was greater when the two rings were independent than when they were fused. In the group of thiazoles, however, there was little to choose between the two types of structure
- 5 Although numerous derived phenols were examined, none was found to compare with the carbamates, some of the latter were even more active than the parent phenols. The value of phenolic carbamates, already well known, has been confirmed, and an unusually high order of potency has been demonstrated in 2-hydroxydiphenyl carbamate. Several groups of derived phenolic ureas and amides were mert
- 6 Among the thiazoles examined none showed much promise of useful potency, but among the pyridines an outstanding order of activity was revealed in 4-benzylpyridine and, more especially, in 2-2'-dipyridyl and in 4 5-phenanthroline The

linkage in the last two compounds and in the corresponding tripyridyl possesses properties which, so far as the experiments have gone, seem to be unique and to offer considerable possibilities as a new starting point in the search for new and highly efficacious anthelminthics

- 7 No activity was discovered among an assort ment of microbial antibiotics In particular, there is at present no reason to think that penicillin or the sulphonamides can yield new anthelminthic agents of any practical value
- 8 The results reported here are at variance with those of earlier investigators in almost every respect, especially in the lactone field. This is attributed to the use by our predecessors of unsuit able methods of in vitro testing. The importance of using experimental material of nematode origin as the basis of methods of this kind is strongly emphasized and its necessity is we believe, confirmed by the general outcome of this investigation

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9 The possibility that some of the substances tested may act otherwise than on the neuromuscular systems of the nematode has not been excluded

The author wishes to record his deep and sincere thanks to Prof E Friedmann, whose chemical skill and co-operation made this work possible suggestions, criticisms and encouragement went far towards aiding the completion of the experiments described here Thanks are due also to Miss L A Norris, Miss M Cotton, and Miss V Moyle, all of whom gave invaluable assistance at various stages To the Agricultural Research of the investigation Council, who provided financial support for the work, and numerous friends who kindly presented samples of a variety of drugs, thanks are also offered Finally, grateful recognition is made of the unfailing courtesy and kindness of the manager of the St Edmundsbury Co-operative Bacon Factory, who arranged the regular supply of living nematode material at all times of the year, often under very difficult conditions

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ACETYLCHOLINE ANTAGONISTS A COMPARISON OF THEIR ACTION IN DIFFERENT TISSUES

BY

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The work of Dawes (1946) on quinidine substitutes brought out points of resemblance which these substances possess apart from their action in cardiac tissue. Quinidine substitutes were found to include some local anaesthetics, some analgesic substances and some spasmolytic substances Dawes pointed out that the local anaesthetic procame possessed as much as 80 per cent of the action of quinidine on the refractory period of the isolated auricle, and that in addition it acted like atropine in diminishing the effect of acetylcholine on the isolated intestine. He also showed that procaine diminished the action of acetylcholine on the rate and force of the heart beat The analgesic pethidine was found to have 83 per cent of the action of quinidine on the auricles, while the spasmolytics papaverine, syntropan and trasentin-6H had 50, 130 and 63 per cent respectively of this action

I have therefore taken a group of seven substances, four of which are local anaesthetics procaine, cocaine, amethocaine and nupercaine, the others being quinidine, trasentin-6H and atropine, and have compared them for their activity as local anaesthetics, for their action in modifying the stimulant action of acetylcholine (a) on the frog rectus and (b) on rabbit intestine, for their action in modifying the depression which acetylcholine produces in the rate and force of the heart beat, and finally for their power to modify the constrictor action of acetylcholine in the blood vessels of the rabbit's ear The purpose of the comparison was to see whether they all possessed the same action towards acetylcholine and how closely the relative potency of these substances in one respect resembled the relative potency in another

Local anaesthetic potency

The local anaesthetic potency of each substance has been tested, using the method described by Bullbring and Wajda (1945) of intracutaneous injection in guinea-pigs

The relative local anaesthetic potency of these substances (taking 1 as the value of procaine) is shown in Table I

TABLE I LOCAL ANAESTHETIC ACTIVITY

Substance	Concentration producing the same effect g /100 ml	Potency
Procaine Cocaine Amethocaine Nupercaine Quinidine Trasentin 6H Atropine	0 46 0 062 0 060 0 047 0 30 0 46 0 92	1 0 7 4 8 0 10 0 1 5 1 0 0 5

There is nothing surprising in the values obtained for cocaine, amethocaine and nupercaine Quini dine might' be expected, like quinine, to have a local anaesthetic action, but not be so strong as to exceed procaine in potency, as it does. It is surprising that trasentin-6H is equal to procaine, and that atropine has as much as 50 per cent of the action of procaine, both are stronger local anaesthetics than was expected.

A comparison of these substances in modifying the situaliant action of acetylcholine on the frog rectus

The isolated frog rectus muscle was suspended in a bath containing 7 ml of frog-Ringer solution. The fluid was replaced by Ringer solution containing 10 ⁷ acetylcholine every 5 min and the stimulant effect of this substance on the muscle was recorded for 90 sec , after washing out with normal Ringer the muscle relaxed to its previous extent. The response to this concentration of acetylcholine was observed at least three times at the beginning of each experiment and found to be the same. Then, 90 sec before the next addition of acetylcholine, the fluid was changed to one containing a solution of the substance to be tested.

its own effect upon the muscle was recorded for 90 sec, the action of this substance in modifying the stimulant action of acetylcholine was then determined. It was found that all seven compounds diminished the action of acetylcholine in a suitable concentration. In Fig. 1 (a) is shown the action of amethocaine added to the bath in a

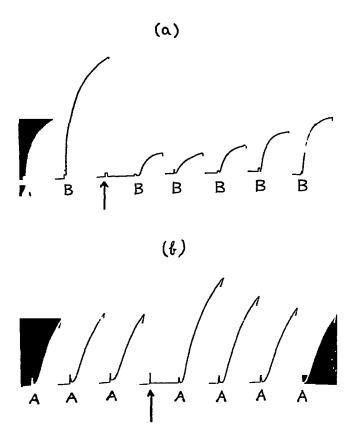


Fig 1 -Frog's rectus muscle preparat on

- (a) A, stimulant effect of acetylcholine added to the bath in a concentration of 5×10^{-8} B, the effect when the acetylcholine added to the bath was in a concentration of 10^{-7} At the arrow, amethocaine in a concentration of 0.5×10^{-4} was added to the bath. The record shows that the acetylcholine effect was greatly diminished
- (b) A, stimulant effect of acetylcholine added to the bath in a concentration of 10⁻⁷ At the arrow trasentin-6H was added in a concentration of 10⁻⁷ The acetylcholine effect was increased

concentration of 0.5×10^{-4} The record shows that the acetylcholine effect was greatly diminished for more than 20 min. When atropine and trasentin-6H were studied, an additional effect was observed, namely, that in a low concentration both these substances increased the action of acetylcholine. This is shown in Fig. 1 (b) for trasentin-6H in a concentration of 10^{-7} . Depression of the acetylcholine effect was obtained with

trasentin-6H at 10^{-5} The corresponding figures for atropine were, for augmentation 10^{-8} and for depression 6.6×10^{-5} The results of comparing the different substances on the same preparation are given in Table II, which shows that the potency of the local anaesthetics on the rectus in relation to one another is in the same order as their local anaesthetic potency Quinidine and atropine are also of similar relative potency to procaine, but trasentin-6H is much more potent on the frog rectus than as a local anaesthetic

A comparison of these substances in modifying the stimulant action of acetylcholine on the rabbit intestine

The isolated rabbit's duodenum suspended in oxygenated Ringer at 34°C was used The spontaneous movements were recorded To the bath of 75 ml Ringer usually 4-10 µg acetylcholine was The effect was observed several times added When regular in the same piece of intestine responses to the action of the same concentration of acetylcholine were obtained, the substance to be studied was added to the bath about 15 min before the addition of acetylcholine, the action of the substance on the intestine and its effect on the response to acetylcholine were recorded seven substances diminished the stimulant action of acetylcholine on the rabbit intestine, there was no evidence of any increase of the stimulant action of acetylcholine when different concentrations of these substances were used As shown in Table II it was found that cocaine and amethocaine had the same activity as procaine, nupercaine was 4 times, quinidine 6 times, trasentin-6H 1,500 times and atropine 3,000 times as active as procaine Fig 2 shows a comparison of the inhibitory effect of quinidine and atropine on the response of the intestine to acetylcholine

A comparison of these substances in modifying the depression which acetylcholine produces in the rate and force of the heart beat

The isolated rabbit auricles were suspended in oxygenated Ringer at 29°C and the spontaneous beat was recorded Every 10 min 04 ml of an acetylcholine solution, containing 100 μ g per ml, was added to the 75 ml Ringer, its depressing effect on the rate and force of the auricular beat was recorded After the effect had been obtained the fluid in the bath was changed to allow the auricles to recover It was found that while procaine, quinidine, trasentin-6H and atropine diminished the depression produced by the acetylcholine (Fig 3a), cocaine had no effect (being

TABLE II
RELATIVE POTENCY IN TERMS OF PROCAINE

		lnh	bitory effect on	the action	of the acetylcho	line		
Substance	Frog rec	tus	Intestin	e	Аппс	e	Rab	bit ear
	Concentration	Potency	Concentration	Potency	Concentration	Potency	Dose µg	Potency
Procaine Cocaine	3 3 × 10 ⁵	1030	2 × 10 ⁵ 2 × 10 ⁵	1 0 1 0	5 × 10 ⁶ 5 × 10 ⁶ 1 × 10-4	1 0 nıl	100 40	1 0 2 5
Amethocaine	1.1×10^{-5}	90	2 × 10 ⁵	10	5 × 10-6	opposite effect	100	10
Nupercaine	9 × 10 ⁻⁶	11 0	5 × 10 ⁶	40	5 × 10 °	opposite effect	100	10
Quinidine Trasentin-6H Atropine	$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	2 0 10 0 1 5	3 3 × 10 6 1 33 × 10 8 6 7 × 10 9	6 0 1,500 0 3,000 0	5 × 10 ⁸ 1 25 × 10 ⁷ 1 25 × 10 ⁸	0 1 40 0 400 0	400 10 2	0 4 10 0 50 0

tested in amounts from 0.5 mg to 8 mg in the bath of 75 ml) Nupercaine and amethocaine (0.5 mg each) actually increased the action of acetylcholine (Fig 3b), smaller amounts (from 20 to 200 μ g) had no effect and larger amounts (2 mg) arrested the spontaneous beating of the auricles To obtain the same effect with quini-

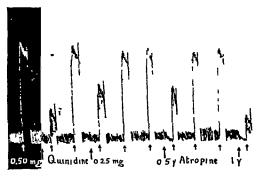
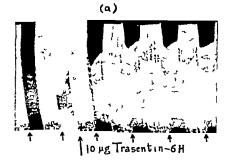


FIG 2—Isolated rabbit s duodenum. The record shows a comparison of the inhibitory effect of quinidine and atropine on the response of the intestine to acetylcholine. At the small arrows 10 µg acetylcholine was added to the bath. The bath was washed out at each interruption of the record. At the first large arrow, 0.5 mg quinidine sulphate was added to the bath, at the second, 0.25 mg quinidine sulphate. At the third 0.5 µg atropine sulphate and at the fourth 1.0 µg atropine sulphate.

dine as with procaine it was necessary to use a quinidine solution 10 times stronger, while the same effect was obtained with a trasentin-6H solution 40 times weaker, and with an atropine solution 400 times weaker, than procaine These results are shown in Table II

The effect of these substances on the constrictor action of acetylcholine in the blood vessels of the rabbit's ear

The carotid arteries of a rabbit under urethane anaesthesia, were dissected and all their side



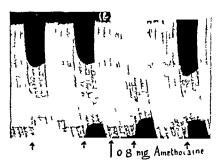


Fig 3 -Isolated rabbit's auricles

- (a) Shows that the depression produced by 40 µg acetylcholine (added to the bath at the small arrow) is greatly diminished after the addition of 10 µg trasentin-6H. The bath was washed out 0.5 min after each addition of acetylcholine
- (b) When 0 8 mg amethocaine was added to the bath the depression produced by the acetylcholine was increased

branches except the posterior auricular artery tied and severed as in the method of Gaddum and Kwiatkowski (1938) The jugular veins were then exposed and the anterior facial and posterior mandibular branches tied, leaving only the branch which drains the ear Cannulae were inserted into both carotid arteries, the jugular veins were tied and incisions made in the veins proximal to these The perfusion was then started ligatures animal was killed and the body detached from the head Glass cannulae were introduced into the jugular veins The outflow was determined by the Gaddum drop timer The injections were made through a rubber cap covering the T-tube connected with the cannulae leading to the carotid The volume of the injections was 0.2 ml The constrictor effect of 100-200 µg acetylcholine on the vessels was recorded on a smoked drum It was found that these substances abolished the constrictor effect of acetylcholine As shown in Table II, procaine, amethocaine and nupercaine had the same activity, quinidine was weaker, cocaine, trasentin-6H and atropine were stronger than procaine These comparisons were made in six different preparations Fig 4 shows an example of this inhibitory effect when cocaine (100 μ g) was used

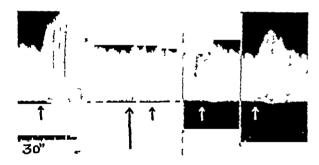


FIG 4—Rabbit's ear perfusion Outflow recorded by Gaddum's drop-timer The record shows the constrictor effect of 200 µg acetylcholine on the vessels (injected at each small arrow), the injection of 100 µg cocaine (at the longer arrow) abolished the constrictor effect of the acetylcholine

Discussion

The suggestion which initiated this work was that different agents, local anaesthetics, spasmolytics and quinidine all act fundamentally as antagonists, direct or indirect, of acetylcholine. It may be wondered why a local anaesthetic should be thought to have this effect. Harvey, Lilienthal, and Talbot (1941), however, record that when acetylcholine is injected intra-arterially it causes very severe pain, and Gray (1947) has shown that the injection of acetylcholine into an artery leading to a detached portion of the skin of the cat sets

up an electrical disturbance in the nerve leaving the skin very similar to that produced by mechanical pressure on the skin Further investigation may therefore indicate that a local anaesthetic is essentially a substance which opposes the action of acetylcholine at sensory nerve endings view is put forward concerning the mechanism of the antagonism, we do not know how, for example, procaine diminishes the stimulant action of acetylcholine on the frog rectus, it may be that procaine blocks the receptors to which acetylcholine must attach itself to produce stimulation, there are many other possibilities of which we are ignorant, and even the conception that the receptors are blocked is little more than a restatement of the observation made

On the whole the investigation has shown that the different substances examined do possess an antagonistic action on all tissues affected by acetylcholine, whether skeletal muscle like the frog rectus, cardiac muscle like the rabbit auricle, or unstriated muscle like that of the intestine or of the blood vessels This, however, does not in itself support the hypothesis mentioned above that local anaesthetics, spasmolytics and quinidine all act fundamentally as antagonists of acetylcholine, since it is probable that all these substances, in some concentration, would also antagonize histamine Histamine is, however, not known to cause pain on intra-arterial injection, or contraction of skeletal muscle, or to have an action comparable with that of acetylcholine on cardiac muscle Hence some significance can be attached to the antagonism of acetylcholine, though there would be much more in a demonstration that the relative potency of these substances in reducing the effect of a natural stimulus was the same as the relative potency in reducing the effect of acetylcholine in that tissue

In different tissues the relative potencies of these substances are very different except that there is a similarity between local anaesthetic action and acetylcholine antagonism on the frog rectus Atropine is 3,000 times more potent than procaine on the intestine, but only 50 times on the blood It is scarcely surprising that these quantitative differences are so great, for they explain the ordinary view that local anaesthetics, spasmolytics and quinidine-like compounds are unrelated in action, and have no common properties the relative potency of the different substances had been even approximately the same on the tissues examined this would have been discovered long The difference, however, does not disprove the hypothesis It is known already that atropine and nicotine vary in their relative anti-acetylcholine action in skeletal and in plain muscle

It is difficult to understand the behaviour of amethocaine and nupercaine on the rabbit auricle In all the concentrations tested they increased the action of acetylcholine and did not depress it, cocaine, moreover, was without action, neither depressing nor augmenting Nupercaine and cocaine are known to modify the action of the isolated heart of the cat, and also to augment the action of adrenaline on the isolated heart (Tripod, 1940), whereas procaine has no such effect is possible that the action of cocaine, amethocaine, and nupercaine in relation to acetylcholine is masked by another effect, the behaviour of these three substances on the auricle may not therefore disprove the rule

SUMMARY

- 1 An examination has been made of the local anaesthetics procaine, cocaine, amethocaine, and nupercaine, the spasmolytics trasentin-6H and atropine, and of quinidine
- 2 These substances have been compared for their local anaesthetic action, and for their power to depress the action of acetylcholine on skeletal muscle (frog rectus), cardiac muscle (rabbit auricle), and unstriated muscle (rabbit intestine and blood vessels)

- 3 The local anaesthetic action of atropine is as much as half that of procaine, while that of trasentin-6H is equivalent to that of procaine
- 4 All these substances depress the action of acetylcholine on the frog rectus, the rabbit intes tine and the rabbit blood vessels. The relative potencies of the four local anaesthetics on the frog rectus are very similar to their relative local anaesthetic potencies.
- 5 Procaine, trasentin-6H, quinidine, and atropine depress the action of acetylcholine on the heart Cocaine is without action, and ametho caine and nupercaine augment the act on of acetylcholine in this tissue

I wish to thank Prof J H Burn for his guidance and supervision throughout this work

This work was done during the tenure of a personal grant from the Spanish Council for Scientific Research

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THE ANTICURARE ACTIVITY OF ESERINE ON THE SUPERIOR CERVICAL GANGLION OF THE CAT

BY

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(Received August 13 1947)

Eserine and prostigmine augment the contractions of skeletal muscle produced by stimulation of the motor nerve. This observation is one which supports the view that acetylcholine plays a part in neuromuscular transmission as both substances are inhibitors of cholinesterase. They are, in addition, powerful antagonists to curare on skeletal muscle

The transmission in sympathetic ganglia is also believed to be effected by acetylcholine, but in the perfused cervical ganglion it is difficult to observe any potentiation of the effects of preganglionic stimulation by eserine or prostigmine. In concentrations from 10⁻⁵ to 10⁻⁴ eserine causes a depression of ganglionic transmission, though Feldberg and Vartiainen (1934) found that a weak eserine solution (10⁻⁶) could be shown to potentiate the response to submaximal and infrequent stimulation as well as that to injection of small doses of acetylcholine

We have now used another method to examine the action of eserine on the ganglion, which is to see if the depression of ganglionic transmission by tubocurarine is relieved by eserine

Метнор

Cats were anaesthetized with pentobarbitone and the superior cervical ganglion was prepared by Kıbjakow's method (1933) as modified by Feldberg and Gaddum (1934) Warm, oxygenated Locke solution was perfused through a cannula in the carotid artery at a pressure around 120 mm of mercury and the venous outflow from the ganglion was collected The preganglionic fibres were stimulated with maximal stimuli at a rate of 8 per second for periods of 15 sec at 3 min intervals. In some experiments the stimulation was continuous so that the contraction of the nictitating membrane was recorded as an uninterrupted plateau All contractions of the nictitating membrane were recorded with an isotonic lever Curare was given as d-tubocurarine chloride Eserine and prostigmine were given as sulphate and methylsulphate respectively

RESULTS

The effect of eserine was observed in five experiments. After recording at least three

maximal contractions of the nictitating membrane, tubocurarine was perfused at a concentration of 1-4 µg/cc Gradually the contractions declined and if no antagonist was injected the response became progressively smaller. When the contraction was diminished by 30-40 per cent the antagonist was injected It was found that eserine in doses of 0 1-0 4 μ g caused an increase of contractions, while doses of 1-5 µg had no effect or possibly caused a further depression illustration of the action of eserine on the curarized ganglion is given in Fig 1 contractions of the nictitating membrane in response to maximal stimulation are shown in (a) After 21 min perfusion with 4 μ g tubocurarine chloride per cc, the contractions diminished to about half the size (b) Now $0.2 \mu g$ eserine was injected 1 min before each stimulation and (c) shows the contractions after the 9th and 10th dose of eserine The effect of tubocurarine was not entirely abolished but the contractions recovered to 86 per cent of their original size When the perfusion with tubocurarine was continued without adding any further eserine, the contractions declined once more to 57 per cent of their original (d) Finally, when Locke solution containing no tubocurarine was perfused, the contractions recovered once more to 86 per cent of their initial size (e) From this experiment it can be seen that the eserine effect is only maintained by repeated injections of 0.2 µg was confirmed in other experiments. The eserine was injected regularly 1 min before s'imulating the preganglionic nerve

It has not been possible to demonstrate any anticurarine action of prostigmine on the perfused ganglion. Repeated doses of 0.05 μg , 0.4 μg , 2.0 μg , and 20 μg were tried in four experiments, but the contractions of the nictitating membrane declined progressively in spite of the addition of prostigmine

The effect of tubocurarine given by single injections of 5 μ g to 10 μ g or by perfusion of a

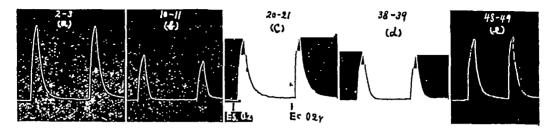


Fig 1—Ganglion perfusion. The numbers above each section are the serial numbers of the contractions of the nictitating membrane.

- (a) Two contractions in response to maximal stimulation
- (b) After 21 min perfusion with 4 μg tubocurarine chloride per c c the contractions diminished to about 50 per cent
- (c) Shows the contractions after the 9th and 10th dose of $0.2~\mu g$ eserine injected 1 min before each stimulation, the effect of tubocurarine was not entirely abolished but the contractions recovered to 86 per cent of their original size
- (d) The perfusion with tubocurarine was continued without adding any further eserine and the contractions declined once more to 57 per cent of their original height
- (e) When Locke solution containing no tubocurarine was perfused the contractions recovered once more to 86 per cent of their initial size

solution containing 4 μ g per c c was also observed during a sustained contraction of the nictitating membrane produced by continuous preganglionic stimulation. When the tubocurarine had reduced the height of the plateau to about 50 per cent of its original height, eserine or prostigmine was injected, but neither eserine nor prostigmine had any antagonistic action under these conditions

The effect of eserine methodide was observed in one experiment After recording three maximal contractions of the nictitating membrane, tubocurarine was perfused at a concentration of 2 µg per c c Gradually the contractions declined and the responses became progressively smaller When the contraction was diminished to 32 per cent, eserine methiodide was injected 1 min before each stimulation It was found that doses of $0.2 \mu g$ and $1.0 \mu g$ had no effect, while doses of 50 μg caused an increase of contractions which recovered to 51 per cent of their original size after the fourth dose of 50 µg of eserine methodide When the perfusion with tubocurarine-was conwithout adding any further eserine methiodide the contractions declined once more to 27 per cent of their original height, after this injections of 25 μ g of eserine methodide 1 min before stimulation had no effect Finally. when Locke solution containing no curarine was perfused, the contractions recovered once more to 67 per cent of their initial size From this experiment it can be seen that the anticurare effect of eserine methiodide is only maintained by repeated injections of 50 µg

DISCUSSION

According to Schweitzer, Stedman, and Wright (1939) the difference between the action of eserine and prostigmine on the spinal reflexes is due to the difference in the chemical structure of the basic nitrogen radicle rather than to their difference in They found that anticholinesterase activity eserine caused an excitatory action of the spinal cord whereas prostigmine caused an inhibition Eserine methiodide, a quaternary derivative of eserine, also produced an inhibition explained the difference between the effect of these substances by their different relative solubility in water and in lipoid It is well known that a quaternary ammonium salt such as prostigmine produces a cation which is soluble only in water On the other hand, the salt of a tertiary ammonium base, such as eserine, on hydrolytic dissociation produces a free base in addition to the cation, and this free base will be soluble in lipoid We found that eserine, if administered in small doses and by repeated injections, had anticurarine activity, while prostigmine had no such action changing the tertiary ammonium salt, eserine, into the quaternary salt, eserine methiodide, its anti curarine activity became 25 times less than that of eserine itself. Since the spinal reflex involves a synaptic transmission not very different from that occurring in the ganglionic synapse, it is possible to explain by this hypothesis the difference between eserine, eserine methiodide, and prostigmine which has been found in our experiments The fact that eserine when given in

large doses or when given during continuous electrical stimulation, no longer has anticurare action, does not contradict the foregoing assumption. Under such conditions there is usually an excessive accumulation of acetylcholine, which, being a quaternary ammonium compound itself, is quite sufficient to cause depression of the ganglion cells.

The picture may, however, not be so simple Bulbring and Burn (1941) have found that eserine and prostigmine were equally depressant to the knee jerk, and both increased the flexor reflex. They believe that there is only a quantitative difference between the action of these two drugs on the reflexes, eserine being more potent than prostigmine

SUMMARY

Eserine antagonizes the action of tubocurarine on transmission in the perfused superior cervical ganglion

Eserine methiodide has an anticurare action on transmission in the perfused superior cervical

ganglion, its relative potency to that of eserine is about 25 times weaker

No such antagonistic effect could be observed with prostigmine

We wish to thank Prof J H Burn and Dr E Bulbring for their direction and encouragement The authors are most grateful to Dr H R Ing for preparing the eserine methodide used in this investigation

The work described above has been done by one of us (T C Chou) during the tenure of a scholarship from the British Council, and by the other of us (F J de Elío) during the tenure of a scholarship awarded by the Consejo Superior de Investigaciones Cientificas in Madrid

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THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON THE TOXICITY OF BAL

BY

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(Received July 21 1947)

Peters, Stocken and Thompson (1945) have already described the steps leading to the manufacture of BAL (2 3-dimercaptopropanol) the specification for which required that BAL should pass a toxicity test before being used for therapeutic purposes

Groups of rats used as standard controls in assaying samples of BAL have shown a wide variation in response to intramuscular injections of 140 mg per kg of Oxford Standard BAL—the LD50 dose as found at Porton (Boyland and McDonald, 1943) Variations from 15 to 85 per cent mortalities occurred in different groups receiving this dose in assays made over several months Such a wide difference in response was greater than would be expected by chance and some other external factor was suspected of contributing to the toxic effects of BAL.

The effects of toxic agents on rats are influenced by many factors (cp. Holck, 1942) among which the most notable is the room temperature at which the rats are kept during the experiment

Temperature being the greatest variant to which Porton rats were subjected, experiments were conducted to see if this affected the mortality of rats to a standard dose of BAL

PROCEDURE

Rats weighing between 120-180 g were starved, but given water, for 24 hours prior to injection of BAL and kept in thermostatically controlled chambers at various temperatures for the whole of the starving period and until 72 hours after injection. Oxford Standard BAL was diluted with propylene glycol to give a concentration of 140 mg/c c. Using a micrometer syringe 1 c c/kg was injected into the muscles of the thigh of each rat and the mortality rate observed for 72 hours after injection. Most of the deaths occurred within 24 hours and only an occasional rat died after that period. For approximately 20 minutes, the average time taken to weigh and inject a group, the rats were exposed to ordinary room temperature.

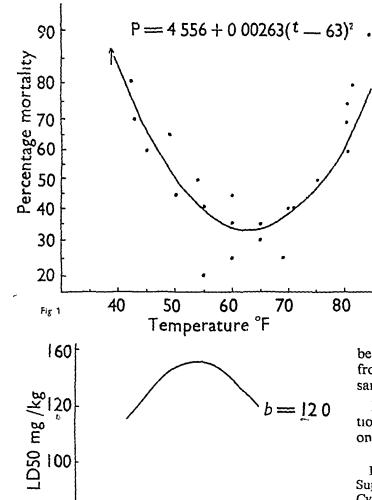
RESULTS

The results obtained over the period between August, 1944, and May, 1945, are given in Table I

TABLE I

D .		Mortali	ty rate
Date	Temperature ° F	Actual	Per cent
29/8/44	39	10/10	100
16/10/44	39	10/10	100
19/10/44	42	8/10	80
1/11/44	43	7/10	70
1/5/45	45	12/20	60
27/4/45	49	13/20	65
1/5/45	50	9/20	45
9/10/44	54	10/20	50
2/10/44	55	8/20	40
24/4/45	55	4/20	20
19/9/44	60	5/20	25
25/9/44	60	7/20	35
17/4/45	60	9/20	45
29/8/44	65	3/10	30
31/8/44	65	7/20	35
24/4/45	69	5/20	25
27/4/45	J 70 J	8/20	40
2/10/44	j 71 j	8/20	40
25/9/44	75	10/20	50
17/4/45	75	10/20	50
31/8/44	80	14/20	70
19/9/44	80	12/20	60
1/5/45	80	15/20	75
9/10/44	81	16/20	80
29/8/44) 84 }	9/10	90
1/11/44	84	18/20	90

Fig 1 shows the same results plotted on probit scale with the best fitting curve, a parabola, symmetrical about the temperature 63° F as calculated by Box (1945) This demonstrates quite a remarkable effect of temperature on toxicity. In view of the fact that variations from the curve are no more than could be expected to occur by chance, there is no evidence that seasonal variation occurred over the period that observations were made other than that due to temperature difference. Fig 2 shows the expected variation of the LD50 for BAL at different temperatures, calculated from Box s BAL-



Temp °F emperature-toxicity formula - LD50 = 152 $(t - 63)^2$, where $t = \text{temp in } ^\circ F$ (Box, 1945)

50 60 70 80 90

Propylene Glycol

Fig 2

30 40

When propylene glycol is used for diluting BAL prior to injection, care should be taken to ensure

that it is freshly distilled We found, when using an old sample of propylene glycol in error, that we obtained much higher mortality rates than were expected in view of the previous results However, when the sample was redistilled, the mortality rates were in keeping with the previous findings. These high results have therefore been discarded and our practice now is to distil off enough propylene glycol for an assay on the same day that the rats are injected

CONCLUSIONS

The mortality rate of rats injected with BAL has been shown to be influenced by the temperature of their environment mortality was minimal at 63° F (172° C) Rats used for BAL assay should be kept at an even temperature or in a thermostatically controlled room if possible No estimation of the relative toxicity of samples of BAL should

be made without reference to the results obtained from a dose of a standard preparation given at the same time

If propylene glycol is used as a vehicle for injection, it is preferable to distil off the required amount on the same day

I am indebted to the Chief Scientist, Ministry of Supply, for permission to publish this paper, to Miss Cynthia Moore for technical assistance, and to Mr G E P Box for evolving a formula from data in Table I

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THE TOXICOLOGY AND PHARMACOLOGY OF METHYL FLUOROACETATE (MFA) IN ANIMALS, WITH SOME NOTES ON EXPERIMENTAL THERAPY

B

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(Received July 23 1947)

It has been known for about ten years that methyl fluoroacetate (MFA), CH₂F COOCH₃, was a substance of great toxicity, with very interesting pharmacological properties. In 1942, Briscoe, and Feldberg, Kilby, and Kilby found that it caused death by convulsions and respiratory failure. Later in 1943, Kilby and Kilby studied it and allied substances further and concluded that the toxicity was due to the CH₂F group

In this paper further work is described on its pharmacological and toxicological properties

MATERIAL AND METHODS

The sample of MFA employed had bp 104°C and sp gr 1 17 It was a clear, colourless liquid with a faint fruity smell, it mixed readily with water, and in dilutions which were toxic, it was tasteless and quite odourless

Animals—In addition to mice, rats, guinea-pigs and rabbits, the larger animals, cats, dogs, monkeys and goats were used for this investigation, also a horse

Toucity—Experiments were conducted with each species and the drug was administered by mouth or by injection as freshly made solutions in distilled water or saline, or by inhalation. For the latter the substance was vaporized rapidly by heat in a 10 cum chamber. Chemical analyses showed that there was little variation in concentration. After administration the animals were observed carefully and records were made of their behaviour over the whole period until their death.

As this substance had a convulsive action the effects of other known convulsants were compared in the most favourable species, and for purposes of record and study a colour film was made of a dog and two monkeys after MFA, and also of monkeys after nicotine, strychnine, and metrazol The effect of MFA on a spinal monkey was also filmed

Autopsies were made on animals directly after death. The effect of MFA on the blood chemistry was investigated in rabbits, dogs, and goats. Blood samples were collected under paraffin, and serum and plasma were separated shortly after. Samples of blood were taken into oxalate tubes for blood.

sugar, non-protein nitrogen, potassium, calcium, and inorganic phosphate

Haemoglobin was estimated by the Haldane carbon monoxide method, plasma proteins and non-protein nitrogen by micro-Kjeldahl digestion and nesslerisa tion (Wong, cf Peters and Van Slyke, 1932), potassium by the micro-cobaltinitrite method of Kramer and Tisdall (cf Peters and Van Slyke, 1932) calcium by the method of Kramer and Tisdall (cf Harrison, 1930), sugar by Hagedorn and Jensen's method (Peters and Van Slyke, 1932), chloride by Sendroy's method (1937), and inorganic phosphate by the method of Obermer and Milton (1932) adapted for use with a "Spekker" absorptiometer

Kymographic tracings of carotid blood pressure and respiration were obtained on cats anaesthetized with sodium barbitone, respiration being recorded by Gaddum's technique (1941), which measures the volume of air breathed in litres per minute

Observations of the clinical effects of MFA were made on both spinal and decerebrated cats, and on a monkey, and kymographic records were obtained of the knee-jerk in spinal, decerebrated and chloralosed cats by means of an electrically operated patellar hammer, in the spinal cat the action of MFA on the threshold stimulus of the flexor reflex (tibialis anticus) was also investigated (Sherrington and Liddell, 1929)

A series of rats were given electrical convulsions on two successive days, and the electrical convulsive threshold was again measured one hour after a sub cutaneous injection of MFA. The apparatus used for human electric convulsive therapy was employed (Golla Walter, and Flemming, 1940)

Finally, various therapeutic measures were attempted, both before and after administration of MFA, and before and after onset of convulsions dogs were mostly used for these experiments

RESULTS

Toxicity by injection by mouth, and by inhalation
The approximate LD50 doses are summarized in Table I It is fully realized that the figures

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for guinea-pig, cat, dog, goat, and monkey are based on evidence from an inadequate number of animals and do not give an accurate assessment of the LD50 doses, the data, however, are suffi-

TABLE I TOXICITY OF MFA

			Oral		Su	bcutar	ieous
Anımal	Dose mg/kg	Mor- tality	%	Approx LD50 mg/kg	Mor- tality	%	Approx LD50 mg/kg
Viouse	15 10 8 7 6 5 4 2	5/5 2/5 0/5	100 40 0	6–7	9/10 9/10 10/10 10/10 5/10 0/10 0/10 0/10	90 • 90 100 100 50 0 0	5
Rat	6 5 4 3 2	5/5 5/5 1/5 0/5	100 100 20 0	3–4	10/10 20/20 20/20 10/10 2/10	100 100 100 100 20	2-3
Guinea- pig	5 3 2 1 0 6 0 5 0 4 0 25	1/1 1/1 1/1 1/1 0/1	100 100 100 100 0 100	0 4	1/1 1/1 2/2 1/1 1/1 0/2	100 100 100 100 100	0 2
Rabbit	10 5 4 2 1 0 5 0 2	2/2 6/6 2/2 2/2 1/2	100 100 100 100 50	0.5	2/2 4/4 2/2 6/6 4/5 1/4 0/1	100 100 100 100 80 25	0 5-1 0
Cat	10 1 0 1 0 3	1/1 1/1 1/1	100 100 100	<03	1/1 1/2	100 50	0.3
Dog	1 0 0 3 0 1 0 05 0 02	1/1 3/3 1/3 0/1 0/1	100 100 33 0	0 1-0 2	5/5 1/1	100 100	0102
Goat	3 0 1 0	1/1	100 100	<10	2/2 2/2	100 100	<10
Monkey	12 10 3 15	2/2 1/2 0/1 0/2	100 50 0	10-12	1/1 0/1	100	10-12

cient to indicate a marked species variation in toxicity without the more wholesale sacrifice of experimental animals which would have been involved in arriving at more accurate figures One horse was injected with 15 mg MFA/kg, which proved fatal

The toxicity by inhalation was investigated more fully in the rat and the mouse than in other animals, the LD50 for rats was 450 mg/cu m for 5 min and for mice above 1,000 mg/cu m for 5 min., 332 rats and 280 mice were used

Skin absorption is of little importance as no deaths occurred in a small series of guinea-pigs with doses up to 100 mg/kg on the plucked skin of the abdomen

Pharmacological effects

There is some difference in detailed behaviour of the species examined, after poisoning with MFA, just as there is a difference in lethal dose, but for the sake of brevity these will be described in groups

- (a) Mice, rats, and guinea-pigs—After a lethal dose by injection there is a delay of about 15 minutes to 2 hours before the onset of symptoms. The animals then become quiet and limp and at the same time rather apprehensive, a stage of hyper-excitability follows, when the animals may jump a foot or more or rush wildly around their cage in circles, tonic convulsions then occur with intervening periods of dyspnoea and flaccidity. Repeated fits are usual and the animal may die either during a fit or in the flaccid interval
- (b) Rabbits—These show a similar period of delay, followed by progressive muscular weakness with gasping infrequent respiration Convulsions start suddenly and death soon occurs
- (c) Cats —After a lethal dose (1 mg/kg) there is a period of about 100 minutes before the onset of symptoms, which are initiated by retching and vomiting, even after injection. The paretic stage follows with acceleration of respiration, incontinence, inco-ordination, and inability to move the limbs. Eye movements are normal, pupils equal but large and they react briskly to light. Pinna and conjunctival reflexes and knee jerks are brisk and there is some knee clonus. Convulsions develop suddenly after about 150 minutes. These tonic fits occur at intervals of about 5 minutes and finally death ensues in about 230 minutes. The heart continues to beat feebly after respiration fails.
- (d) Dogs—The latent interval is only about 30 minutes after 0.3 mg/kg. A quiet period is followed by hyper-excitability with loud barking and wild, inco-ordinate, impulsive activity, associated with incontinence of urine and faeces Retching and vomiting may occur before this stage. The excitable stage suddenly merges into

one of convulsions At first tonic and extensor, with dilated pupils and brisk reflexes, later the fits are clonic, with champing of the jaws and inco-ordinate arrhythmic running movements of all four limbs. After a brief respite with heavy panting, the whole pattern is repeated at intervals of 1-2 minutes until death occurs, once again from respiratory failure.

(e) Goats and horses—These animals show a latent period followed by weakness, collapse, tachypnoea, cold sweating in the horse, a marked fall in temperature, rapid pulse and death from respiratory failure—In neither animal were convulsions seen

(f) Monkeys—The monkeys (Macacus Rhesus) though less susceptible, are most interesting After a latent interval of about one hour, with a dose of 10 mg/kg by mouth, they become quieter, not taking much notice of their surroundings Retching and then vomiting, even after injection of the poison, are usual In about an hour the pupils are dilated After 70 minutes one such animal was sitting up with pallid face, its head turned looking over the right shoulder defaecated incontinently and after a few minutes its head was rotated again to the side and there were coarse jerky tremors of the head symptoms followed at intervals of a few minutes and conjugate deviation of the eyes was seen in conjunction with the head rotation It appeared very dazed and suddenly fell over and started convulsions, which were tonic at first and then clonic It looked strangely round and made no attempt to escape after this convulsion. A similar seizure was seen in a few minutes, initiated once again by head rotation and conjugate deviation of the eyes to the right side An asymmetrical spread of the convulsion to the right side was followed by a generalized tonic fit, succeeded by clonic spasms During the asymmetrical stage there was a onesided risus sardonicus, slight head retraction, glabellar spasm and the knee jerks were accen-This spasm of the frontalis muscle was a prominent symptom accompanying the upward and outward eye deviation. The tonic stage usually lasted 30-40 sec and the clonic 2-3 min and these were succeeded by a relaxed phase when the animal appeared semi-conscious and exhausted, even prostrate Repeated convulsions may progress to full unconsciousness and death, or there may be a cessation of fits followed by a slow recovery, the animal finally sitting up in an inco-ordinate and dazed manner After some hours it will seem little the worse, having regained full activity and appetite During the whole of

this period monkeys are silent, but their expression conveys firstly an apparent headache, then fear, anxiety, and distress, and finally loss of appreciation of their plight

Comparison of MFA with other convulsant drugs

A detailed comparison was made of the effects of MFA with those of nicotine, picrotoxin, strychnine, and leptazol in intact cats, monkeys, and rats and it was quite clear that MFA resembles leptazol more closely in action than it does nicotine or strychnine, but its effects develop much later and last much longer Electrically induced convulsions in the rat were identical in appearance with the MFA tonic fit

Cumulative action

MFA is apparently not entirely excreted or detoxicated within 24 hours, and if doses below the convulsant threshold are given daily to dogs some cumulative effect is seen

Dog 28, wt 59 kg, was given 0 025 mg/kg (1/4 of the lethal dose) by mouth for five days. It was completely unaffected until after the fifth dose, when convulsions started and death ensued

Administration of bigger sub-convulsant doses, how ever, can continue with impunity on alternate days or less frequently, and either the animal is unaffected or death occurs if the dose is raised to the lethal. This again suggests that there is little tolerance to repeated and increasing doses

.Dog 33, wt 91 kg, was given 0 025 mg/kg every third day by mouth in 30 days without any symptoms at all

Dog 63, wt 12 kg, was dosed as shown in Table II and death occurred after the usual lethal dose of 0.1 mg/kg had been reached

TABLE II

Date	Dose, mg /kg	Dose, mg
22 June	0 05	0 6
24 ,,	0 05	06
26 ,,	0 05	0 6
28	0 05	06
30 .,	0 08	10
2 Iuly	0 08	10
24 ,, 26 ,, 28 ,, 30 ,, 2 July 5 ,	0 10	1 2
Total	0 46	5 6

Post-mortem appearances

In the animals dying after a long series of severe convulsions, signs of asphyxia were found Often the animal died in the position of a tonic extensor fit with rigid limbs and tail, bulging livid eyes and cyanosed tongue The blood was thick and dark in colour and the veins and the

TABLE III

Time Condition	Нь%	g /100 ml		mg /1	00 ml plasr	na		
Time	Condition	10 _{/0}	Plasma protein	Non- protein N	K	Ca	Cl	Inorg PO ₄
Before 90' 125'	anaesthetized preconvulsive after convulsions	64 5 89 0 103 0	6 0 6 8 6 9	25 25 30	18 7 22 6 47 8	10 2 8 4 12 8	387 406 414	3 6 2 45 6 40

right side of the heart were distended. The ventricles were usually contracted. All the organs were congested and dark, especially the liver, but the spleen was small, dark, and contracted. The lungs were bluish, showing congestion, areas of collapse, and emphysema, and the bronchi often contained frothy fluid, but there was no oedema. Sometimes petechial haemorrhages were seen in pleura and endocardium. The brain and meninges were usually congested. Goats did not show convulsions and there was little to find except congestion. The kidneys and other organs did not reveal anything of interest.

Effect on the blood chemistry

Blood sugar —It was suggested that MFA might cause hypoglycaemic convulsions, but investigation of two rabbits and a dog after large doses, many times lethal, revealed if anything a rise in blood sugar, as one might expect in a condition associated with convulsions and hypoxia

Table III shows the effect of MFA (2 mg/kg) by subcutaneous injection in a dog under light nembutal anaesthesia

It was thought that the considerable rise in serum potassium might be due to the convulsions and asphyxia, and so similar investigations were made on two goats without an anaesthetic (Table IV)

The rise in non-protein nitrogen and serum potassium was significant in each experiment, but only as a terminal event. In the dog the greater rise was probably due to the severe muscular convulsions. Calcium showed a slight fall in the goats, but this was not sufficient to cause tetany. The considerable haemoconcentration noted in all animals was not accompanied by an alteration in plasma proteins, and it might be explained by an outpouring of erythrocytes from the spleen, which at autopsy was always empty. Again, in the goats there was a considerable rise in inorganic phosphate

Effect of MFA on decerebrated and spinal animals

Decerebrated cats after 5 mg/kg IV—Extensor rigidity was enhanced, the forelimbs were hyperextended and the head retracted Respiration was stimulated in rate and depth Convulsions began within 25 min of injection, continuing at intervals Swallowing movements were seen after 25 min, followed later by retching Respiration began to fail, but by applying artificial respiration the heart was kept beating for several minutes

Spinal cats after 4 mg/kg IV—After 60 min all reflexes were more brisk and the knee jerk was followed by irradiation of stimulus until finally convulsions started Pinching of the foot-pad stimulated a mass reflex and then convulsions, and these continued, clonic and occasionally tonic, intermittently

TABLE IV

			g /100 mJ	mg /100 ml plasma					
Time	Condition	Нь%	Plasma protein	Non- protein N	К	Ca	CI	Inorg PO₄	
GOAT 506 Before 40' 110' 160'	normal collapsed and dyspnoeic distressed and tachypnoeic moribund	56 5 66 0 68 0 72 3	6 8 6 1 6 4 6 5	27 31 31 36	17 2 19 1 18 8 25 6	9 9 9 4 9 1 9 0	309 338 328 328	3 72 6 75 7 65 8 75	
GOAT 507 Before 40' 110'	normal collapsed and dyspnoeic moribund	66 0 77 5 82 0	6 8 6 2 6 5	35 35 39	14 1 17 3 22 0	9 8 9 1 8 6	364 356 366	4 80 6 55 8 30	

until the heart stopped three hours after injection. In another cat given 2 mg/kg, spontaneous movements of the forelimbs and twitching of the dartos muscle and penile retraction were seen.

Spinal monkey—After an initial phase of spinal shock lasting 2½ hours, MFA was given, and during the course of 4½ hours a total dose of 100 mg/kg (10 times the normal intact lethal dose) was injected

Irradiation of excitor stimuli was more pronounced Chest stimulation by tickling evoked movements of the limbs and trunk, and gentle upward pressure in the lumbar region with two fingers caused active contraction of abdominal muscles to take the weight of the lower half of the body. The tail twitched continuously. Later there were spontaneous twitching movements of the rear toes, but no actual convulsions occurred in this preparation as in the spinal cat

Blood pressure and respiration of anaesthetized cats

Doses of MFA lethal in 18 hours, i.e., 0.3 mg/kg, had no effect on the BP or respiration, but when the dose was increased to 2.9 mg/kg, there was a small but gradual rise in pressure. Respiration was stimulated and the volume in litres per minute increased, but after a total dose of 9 mg./kg it became gasping in type, getting slower and more spasmodic.

Comparison was also made with acetylcholine (Fig 1) before and after atropine (both muscarine

respiration ceased, the heart continued beating for about 5 min more, while the blood pressure fell rapidly

Knee jerk

Chloralosed cat (Fig 2)—There was increased extensor tone and the jerk was brisker and the lever did not return to its original base line. Later the reflexes diminished for a while and the animal started generalized convulsions and respiration failed

Decerebrated cat (Fig 3)—Patellar stimuli evoked irradiation of reflex responses, but the knee jerk was not apparently increased Extensor rigidity was accentuated Convulsions started and respiration failed

Spinal cat—The knee jerk suddenly increased for three or four patellar stimuli, and then further records were impossible as generalized convulsions supervened

Effect on flexor reflex of the spinal cat (Tibialis anticus contraction via reflex arc of popliteal and peroneal nerves) (Fig 4)—After injection of 2 mg/kg MFA, the threshold shock was found at regular intervals. The results are plotted graphically (the distance in cm of the coil against the time in minutes) and show that there is a progressive reduction in threshold, until finally with the coil moved away to 33 cm (normal 24 cm) the tibialis anticus goes into myoclonus. Further records were impossible as the

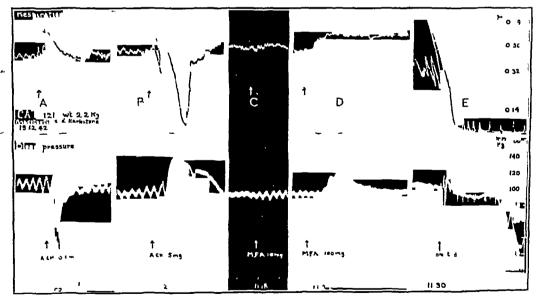


Fig 1—Comparison of acetylcholine and MFA on BP and respiration. At A, 01 mg Ach at B, 5 mg Ach, between A and B, 10 mg atropine sulphate, at C, 10 mg MFA, and at D 100 mg MFA.

and nicotine effects) After atropine 5 mg/kg MFA had no immediate effect on either B.P or respiration, but a very large dose (50 mg/kg) produced an immediate stimulation of respiration and a rise in BP similar to that produced by a large dose of acetylcholine The animal soon began to vomit and

preparation developed generalized tonic and clonic convulsions

Effect on electric convulsive threshold in rats

By varying the voltage or time in 1/10 sec the convulsive threshold was determined on two succes

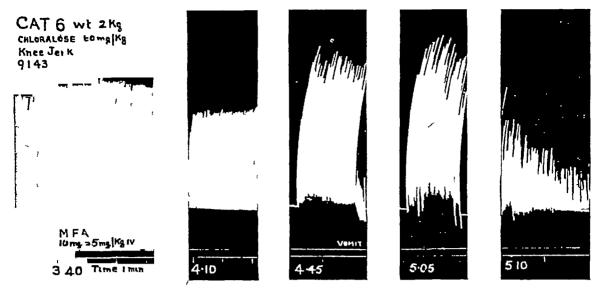


Fig 2 - Effect of MFA on knee jerk of chloralosed cat

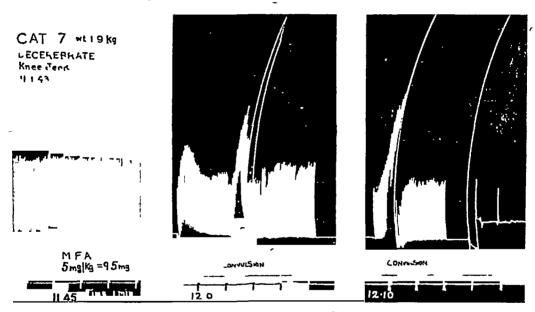


Fig 3 - Effect of MFA on knee jerk of decerebrate cat

sive days in 20 normal rats, and on the third day the threshold was determined again 3/4-1 hour after injection of 5 mg/kg MFA

The results demonstrated quite clearly that the electric convulsive threshold is reduced by at least ten times. The convulsions obtained by electric shock and MFA were identical but the electrically induced convulsions produced in the injected rats were more severe and of longer duration.

Experimental therapy

Convulsive phase—Dog 19, given 1 mg/kg MFA by mouth, suddenly started convulsions after 87 min Attempts were made to inject sodium phenobarbitone, but these failed and chloroform was administered on an open mask. Although the seventy of the tonic and clonic spasms was reduced the convulsions con-

tinued and the dog died before full anaesthesia was obtained /

Preconvulsive stage—(1) Dog 53 was given 0.3 mg/kg MFA sc, and 45 min later 4 mg/kg dilantin (sodium diphenyl hydantoin) was given by mouth As the excitable stage had started after 110 min 40 mg/kg sodium phenobarbitone was injected intramuscularly Convulsions of modified nature began in 120 min, and death followed 10 min later

(2) Dog 57 was injected with 0 3 mg/kg MFA sc, and this was followed by 40 mg/kg sodium phenobarbitone after 115 min at the beginning of the excitable stage. Fits started after 138 min, and another 40 mg/kg sodium phenobarbitone was injected intramuscularly, very mild convulsions rather like gross tremors continued without any

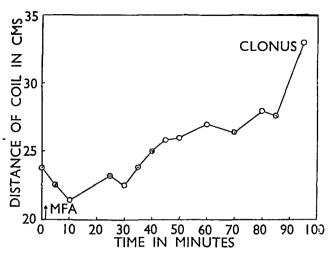


FIG 4—Effect of MFA on flexor reflex (tibialis anticus) of spinal cat MFA 2 mg/kg s c Ordinates distance of coil in cm Abscissae time in minutes

tonic rigidity of the chest or restriction of respiration, but death occurred at 195 min

(3) Dog 58, 03 mg/kg MFA sc After 120 min it was quiet and was given dilantin 10 mg/kg by mouth, but convulsions started at 150 min Then 2 mg/kg omnopon (equivalent to 1 mg/kg morphine) was injected intramuscularly There was no benefit, and it died at 165 min

(4) Dog 54, 03 mg/kg MFA sc The first 105 min were symptom free, 2 mg/kg omnopon was then injected The dog was partly narcotized at 140 min, when the excitable stage started and another dose of omnopon (2 mg/kg.) was given Although the dog became quieter, convulsions occurred at 180 min, but were modified by the morphia It died at 225 min

(5) Dog 55, 0.3 mg/kg MFA sc After no symptoms for 120 min, hyoscine hydrobromide (0 02 mg/kg) was injected and repeated again at 195 min as the excitable stage had begun Controlled and modified convulsions started at 230 min, lasting until 275 min, when death occurred

(6) Dog 53, 0 3 mg/kg MFA by mouth Thirty min later sodium phenobarbitone (20 mg/kg) was given by mouth, and another 20 mg/kg. again by mouth at 90 min This treatment had effectively delayed symptoms by 6½ hours, and the dog, although sleepy, was sitting up and ate a big meal At 7½ hours his pupils dilated and the excitable stage had begun Another 20 mg/kg sodium phenobarbitone was injected intramuscularly and he became quieter. The same dose was repeated again at 8½ hours although he was sleeping. No further anxiety was felt, and the dog remained semicomatose for 30 hours and gradually made a full recovery

Prophylaxis

(1) Dog 34 was given 84 mg/kg methylphenobarbitone (Rutonal M and B) by mouth on one day and

56 mg/kg on the next day, and this was followed by 0.4 mg/kg MFA when the dog was yawning and sleepy

All symptoms were postponed until 6½ hours, when the hyperexcitable stage began. He was then given 0.35 mg/kg tubocurarine chloride intramuscularly Modified fits followed, and continued at intervals of about 3 min. The knee jerk was still present after 20 min, but respiration failed and death followed at 6½ hours.

(2) Sodium diphenyl hydantoin (10 mg/kg.) by mouth was given to dog 60 on two successive days, followed by 5 mg/kg on another two days. This dosage made the dog quiet and subdued, 0.3 mg/kg MFA was then given by mouth, but the effect of the anticonvulsant was insufficient to prevent the onset of convulsions after 4 hours. The pattern of the fits was modified, they were shorter, of normal severity, and with less relaxation in between. The dog died after 44 hours.

Other measures to reduce muscular excitability, such as injection of magnesium sulphate and chloride and calcium gluconate, have been ineffective in preventing death in rabbits

Oxygen and CO2

Two batches of 10 rats injected with 5 mg/kg. MFA were used, the one as controls and the other kept in a small chamber in a continuous stream of O₂ and CO₃ for 5 hours. All the control rats had convulsions before those treated but the mortality was as follows

Mortality	Treated	Controls
24 hours	7/10	2/10
36 ,,	8/10	4/10
3 days	10/10	7/10

DISCUSSION

Methyl fluoroacetate is an exceedingly interesting poison because unlike most others it is about as toxic by mouth as by injection. The fact that doses of 0 I-10 mg/kg by mouth will kill all the species of animals tested, except the monkey, mouse, and rat, shows that it is more toxic than strychnine or nicotine and brings it into the category of aconitine and the most poisonous substances known. Lethal concentrations in food, water, or milk were not detected either by smell or taste, even by animals such as the cat or dog with very keen senses.

Investigation of its pharmacological effects in the higher mammals shows that it is a powerful convulsive poison and careful clinical observation and cinematographic analysis of its action in monkeys strongly suggests that it acts predominantly on the cerebral motor cortex and on the rest of the central nervous system to a lesser degree. These suppositions are confirmed by a

comparison of the effects of nicotine, strychnine, and leptazol (metrazol) on the monkey Briefly MFA has almost an identical convulsive pattern with that of leptazol Both show a similar initiation of the convulsion by an apparent asymmetrical stimulation of the premotor cortex, demonstrated by conjugate deviation of the eyes and head rotation followed by a spread, at first unilateral and confined to the same side of the body, and then becoming generalized

MFA however differs very much from leptazol in some respects, whereas the effect of leptazol is immediate, occurring within 5-9 sec after intravenous injection, that of MFA is delayed for some 15-20 min. Further, leptazol usually produces one fit only and is rapidly destroyed in the body, whereas MFA almost invariably causes repeated convulsions either close together, lasting about 15 min and followed by death, or more widely spaced and usually followed by recovery. The lethal dose by mouth and by injection is practically the same and the toxic symptoms appear after a similar time, whether it is given by mouth or by injection. Even intravenous injection has little effect on the time interval

Investigation of the smaller animals led the Cambridge workers to compare its action with that of nicotine, and in some animals, even in some monkeys, the depressive action is sufficient to support this view, but whereas nicotine depresses the knee jerk in monkeys MFA does not and may even increase it. Again, MFA has a nicotine-like effect, raising blood pressure after big doses, but no effect after doses which produce convulsions and even death.

At first we thought its action was like that of strychnine, the type of convulsion in the rat was similar, and in the preconvulsive excitable stage in the cat, auditory stimuli demonstrated a nervous hyper-sensitivity. In the monkey, however, a real difference between strychnine and MFA was seen, convulsions being generalized and symmetrical with the former and asymmetrical to start with in the latter and almost Jacksonian in pattern. In the rat the convulsion is predominantly tonic and identical with that produced by electric shock.

In the monkey the repeated and severe tonic and clonic seizures, together with the conjugate deviation of the eyes, very strongly suggest a resemblance to the condition of status epilepticus in man Evidence in man confirms this belief, and in fact repeated and severe fits indistinguishable from status epilepticus are observed

Although MFA is a powerful convulsant in most animals, suggesting possibly that death is due

to exhaustion and cerebral anoxia after repeated convulsions, there is considerable evidence against such a simple cause of death Goats and horses do not convulse, and observation of these and other animals shows that death is due to a progressive failure of respiration, the heart continuing to beat for some minutes after breathing has ceased addition there is considerable haemoconcentration, but as there is no alteration in plasma proteins or evident oedema it is likely that this is chiefly due to contraction of the spleen and mobilization of red blood corpuscles from other depots Many of the animals become very cold with apparent peripheral circulatory failure, this considerable loss of body heat being well marked in the horse Further investigation on this line may possibly reveal some central effect on the heat regulating centre or mechanism Changes in blood chemistry reported here are based on a very small number of animals only, but they reveal nothing which might be of value in the interpretation of the mode of action or in possible treatment

If MFA were used as a poison for rabbits or rodents and other mammalian pests, there would always be the risk of accidental poisoning in man, especially as it is tasteless and odourless the incomplete evidence of its action available. but with the knowledge of the symptoms and probable cause of death, various therapeutic measures have been attempted The difficulties are great the first indication of poisoning in man is the onset of epileptiform convulsions after an initial period of nausea and mental apprehension, treatment then amounts to very urgent measures to control status epilepticus, and to prevent the developing depression of respiration, there is considerable danger that active attempts to control convulsions by drugs, which themselves may be depressive to the respiratory centre, will only accelerate death

There is some evidence that if sublethal or subconvulsive doses are taken by mouth at daily or less frequent intervals it has a cumulative effect, and this is confirmed by Kilby and Kilby (1943) for inhalation by guinea-pigs, but they suggest that in rats the reverse is true, and that rats develop tolerance to repeated exposure to small doses by inhalation.

The most effective drug for raising the threshold of convulsions in epilepsy and electrically induced fits in man (Hemphill and Walter, 1941) is sodium diphenyl hydantoin (dilantin) According to Golla (1943) this drug does not alter the explosive motor discharge from the cortex in epilepsy, but prevents its radiation through the central nervous system, unlike sodium luminal, which is thought to reduce

the actual motor discharge in the cortex and not to influence the spread. Unfortunately dilantin has a pH of 114 and is only administered by mouth, the full effect taking about 6 days to develop, therefore it is quite ruled out as a method of treatment for poisoning with MFA

Sodium phenobarbitone or sodium luminal can be injected intramuscularly or intravenously, one dog survived three lethal doses of MFA when the barbiturate was administered repeatedly, starting 30 min after the animal had swallowed the poison and before the onset of toxic symptoms, convulsions were prevented and yet fatal depression of respiration did not ensue

The application of intravenous anaesthetics has to be pushed to full doses in order to produce anticonvulsant effects, because animals, even under chloralose or nembutal anaesthesia, still develop convulsions when MFA is given

So far, in dogs, which were the most convenient and the most susceptible animals, no method of treatment has been effective once the convulsive phase or epileptiform state has been reached, and the technical difficulties of therapy, involving intravenous injection into a dog in convulsions, limit other possible lines of treatment However, reviewing one's present knowledge of MFA the following lines of treatment for man are suggested

- 1 Early intravenous injection of a rapidly acting anaesthetic such as pentothal sodium or evipan sodium followed by
- 2 intramuscular injection of a more prolonged acting cortical depressant, such as sodium phenobarbitone or sodium luminal, or rectal avertin,
- 3 very careful supervision of respiration supplemented by adequate oxygen therapy with BLB mask and use of Bragg-Paul and/or Eve methods of artificial respiration,
- 4 possible use of intravenous hypertonic glucose as in status epilepticus,
- 5 careful use of tubocurarine chloride to control convulsions

SUMMARY

- 1 The toxicity of MFA by mouth and subcutaneous injection has been determined for a variety of animals. There is considerable variation of dosage from 0.1 mg/kg in the dog to 10-12 mg/kg in the monkey and the order of decreasing susceptibility is dog, guinea-pig, cat, rabbit, goat, and probably horse, rat, mouse, and monkey
- 2 The toxicity by inhalation for the rat and mouse has been investigated more fully than for other animals the LD50 for rats is 450 mg/cu m

- for 5 min and for the mouse above 1,000 mg/cu m for 5 min
- 3 The pharmacological effects of this substance by mouth and by injection in all the animals investigated are described. In most animals it is a convulsant poison and causes progressive depression of respiration. It is toxic by inhalation, injection, and by mouth, but not when applied to the skin
- 4 The effect of MFA is compared with those of nicotine, strychnine, leptazol (metrazol), picrotoxin, and electrically induced convulsions in the rat, cat, and rhesus monkey The convulsive pattern is considered to be similar to that of leptazol
- 5 Post-mortem findings are briefly described, but little is found except signs of asphyxia
- 6 In a small number of rabbits, dogs, and goats estimations have been made of blood sugar, haemoglobin, plasma proteins, non-protein nitro gen, and serum potassium, calcium, chloride, and inorganic phosphate. Apart from the terminal rise in non-protein nitrogen and potassium, blood changes include a rise of 20–60 per cent in Hb, up to 90 per cent in blood sugar, 70–130 per cent in inorganic phosphate and a less significant rise in serum potassium.
- 7 MFA, like leptazol, acts on the whole central nervous system, but the higher centres are more sensitive than the lower
- 8 Graphic records show that MFA stimulates the rate and volume of respiration and then causes failure of respiration, probably central in origin Blood pressure is little affected by small doses, but very large doses have a nicotine-like action
- 9 MFA appears to accentuate the knee jerk until irradiation of the stimuli is so facilitated that convulsions occur
- 10 Nervous conduction in the reflex arc of a spinal cat is increased and the threshold stimulus lessened
- 11 In rats the electric convulsive threshold is reduced about 10 times by MFA
- 12 As MFA is both a-powerful convulsant and a respiratory depressant, the difficulties of treatment are stressed, but suggestions for treatment in man are made
- 13 Since MFA is about equally toxic by mouth and by injection, and is not readily detected or destroyed, it presents a serious hazard as a food and water contaminant if used as a poison for rodents and other vermin

The chemical estimations of plasma proteins, non protein nitrogen, calcium, and potassium were made by Dr F C Courtice, and estimations of chloride and

inorganic phosphate by K M Wilson, to whom I am indebted

I am also grateful to Professors Cameron, Lovatt Evans, Gaddum, and Golla for their advice and help, also the Chief Scientist of the Ministry of Supply for permission to publish

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QUANTITATIVE STUDIES OF PROCAINE METABOLISM IN THE CAT

RY

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During the past few years there have been references to therapeutic uses of intravenously administered procaine, mostly to produce analgesia (Lundy, 1942, Gordon, 1943, McLachlin, 1945, Allen, 1945) and also to counteract cardiac arrhythmias (Burstein and Marangoni, 1940 Burstein, 1946)

Intravenous administration of a drug as toxic as procaine, whether by a single rapid injection or by a slow infusion, involves a consideration of the rates of dilution, diffusion, fixation, excretion, and destruction of the drug in the body Eggleston and Hatcher (1916) showed that the toxicity of intravenous procaine increased directly with the rate of injection, and Macdonald and Israels (1932) and Hill and Macdonald (1935) demonstrated the same relation for depression of breathing Eggleston and Hatcher (1916) also showed, by perfusion experiments in cats, that procaine is destroyed in the liver, but, using a biological test for the estimation of procaine concentrations, they observed no destruction of procaine by the blood in dogs. These results were confirmed by Dunlop (1935) in experiments on dogs, in which procaine and p-aminobenzoic acid blood levels were determined by a diazotization method, using β -naphthol as coupling reagent, after hepatectomy he still found some destruction of procaine which was attributed to an action by tissues other than the liver, though blood itself appeared to be mactive Recently, however, Goldberg, Koster, and Warshaw (1943), Kisch, Koster, and Strauss (1943), and Hazard and Ravasse (1945) have adapted the Bratton and Marshall (1939) method for sulphonamides to the determination of procame and p-aminobenzoic acid in blood and tissue fluids They have shown that the plasma and serum of many species of animals, including man (in whom the effect is most powerful of all), can hydrolyse procaine to p-aminobenzoic acid and diethylaminoethanol. They ascribe this action to the

presence of an enzyme, "procaine esterase," which is said to be distinct from cholinesterase, tropinesterase, and lipase

Our work was designed to study procaine blood levels in cats after rapid intravenous injection, or slow infusion at various rates, with the special aim of determining the contributions made by diffusion, fixation, excretion, and destruction to the rapid removal of procaine from the blood. The parts played by the liver and kidney and the effects of physostigmine, neostigmine, methylene blue, and disopropylfluorophosphonate on procaine metabolism have also been studied.

Chemical reactions involved in experiments

Procaine is hydrolysed as follows -

$$NH_{3} \longrightarrow CO OCH_{2}CH_{2}N \xrightarrow{C_{2}H_{5}} \rightarrow NH_{3} \longrightarrow COOH + (C_{2}H_{4})_{2}N CH_{2}CH_{2}OH$$

p-Aminobenzoic acid may be acetylated, a reaction which occurs slowly in the liver and which is negligible during the perfusion experiments to be described. The amount of acetylation is consider able in the rabbit and in man, but is small in the cat (Ansbacher, 1944) p-Aminobenzoic acid may also be conjugated with glycine to form p-aminohippuric acid. This reaction is very slight in man and in the cat (Beyer, Mattis, Patch, and Russo, 1945)

METHODS

Cats were anaesthetized with chloralose (80 mg/kg body weight) Arterial blood pressure was recorded by a cannula in one common carotid artery. One femoral artery was cannulated for collection of arterial blood samples. The injections of infusions of procaine were given into the femoral vein of the opposite side. Infusions were kept at a constant rate

by the use of a Marriotte bottle and a drip bulb, into which was incorporated a platinum loop connected to a Gaddum drop recorder to give a graphic record on the smoked drum. In this way changes in rate of flow were soon noticed and corrected. Procaine hydrochloride was made up to 03-05 per cent (w/v) in 09 per cent (w/v) NaCl solution. The rate of administration is expressed as mg of procaine hydrochloride/kg body wt/min

Femoral artery blood samples were collected into a mixture of sodium fluoride and potassium oxalate to give a final concentration of M/2 NaF and M/20 $C_2O_4K_2$ H_2O The blood-oxalate-fluoride mixture was then placed in a refrigerator at -5° C. It had previously been shown that at room temperature less than 5 per cent of procaine was destroyed in 1 hour in contact with this mixture, and on freezing the loss was undetectable. At the end of an experiment 2 c c of each blood-oxalate-fluoride mixture were added to 8 c c of 12 5 per cent (w/v) trichloroacetic acid in order to precipitate proteins and provide an acid medium in which procaine is stable

Procaine and p-aminobenzoic acid concentrations in blood and urine were determined by a modification of the method described by Kisch and Strauss (1943). The procaine was extracted in narrow tubes into chloroform (instead of ether) and re-extracted into 0.6 N HCl in which the procaine concentration was then determined by the method of Bratton and Marshall (1939) as used for sulphonamides. Similar determinations were made on the blood filtrate, directly and after boiling for 1 hour with 6N HCl, these enabled the concentrations of free and acetylated p-aminobenzoic acid to be calculated.

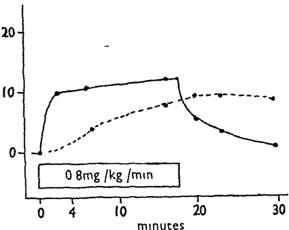
The effect of the liver on procaine destruction was studied by noting the change in blood procaine concentration produced by excluding the liver from the circulation, in a manner similar to that described by Smyth (1947) in his studies on acetate metabolism. The whole of the portal area was excluded from the circulation and the hepatic artery was isolated but left patent. It was found that when a constant rate of procaine infusion had been established for about 15 min the blood level had become constant. The hepatic artery was then clipped so as to cut off nearly all the remaining blood flow through the liver, and the infusion was continued at the same rate.

RESULTS

Continuous infusion of procaine

Procaine infusions have been given at different rates for periods of up to 30 min. At the beginning of the infusions there was frequently a 10-15 mm. Hg rise in blood pressure, but the rates of flow studied did not subsequently reduce blood pressure or depress breathing except with rates exceeding 20 mg/kg body wt/min.

In Fig 1 the effect of an infusion of 0 8 mg/kg/min of procaine for 18 min is shown. The blood



level of procaine rose within 3 min to 95 mg/l and subsequently to 12 mg/l after 16 min. Within 2 min of stopping the infusion the blood level had fallen to 5 mg/l and 12 min after the end of the infusion it was 2 mg/l. The level of p-aminobenzoic acid rose during the course of the infusion to 75 mg/l and after the end of the infusion a further slight rise to 9 mg/l was noted, 12 min after the end of the infusion it was still 85 mg/l

In Fig 2, 25 mg/kg/min of procaine was infused for 125 min At 25 min the blood level was 35 mg/l, at 5 min it was 55 mg/l, and at 12 min it had risen to 66 mg/l. After the infusion was

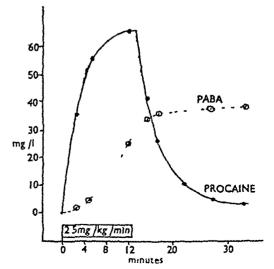


Fig 2—Ordinates and abscissae as in Fig 1 Procaine infusion at 2.5 mg/kg/min Procaine and p-aminobenzoic acid (PABA) blood levels during and after infusion

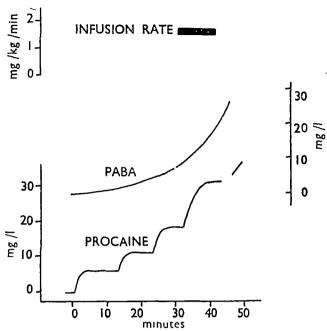


Fig 3—Curves of blood procaine and p-aminobenzoic acid concentrations at different rates of infusion of procaine Upper part of record rate and duration of infusion Lower part of record procaine and p-aminobenzoic acid (PABA) blood concentrations Ordinates on left procaine Ordinates on right p-aminobenzoic acid

stopped the blood procaine level fell rapidly, and within 2 min of cessation it was 40 mg/l, within 5 min it was 25 mg/l, and subsequently it declined more slowly to 3 mg/l by 20 min after the infusion had ended

The effects of successive increases in rates of infusion on blood procaine levels are shown in Fig 3 At rates of 0.5–1.6 mg/kg/min the blood curves flatten out by the end of the infusion. The

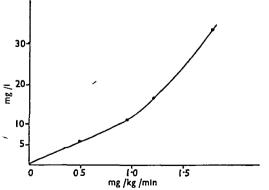


Fig 4—Relation of infusion rate to blood procaine concentration Data from curves of Fig 3 Ordinates concentration of procaine in blood in mg/l Abscissae infusion rate of procaine in mg/kg/min

infusion at 21 mg/kg/min was not maintained long enough to reach a plateau level, owing to the development of respiratory failure. It was found that whenever the infusion had been tolerated for a sufficient period (10–30 min depending on the rate of infusion) a constant blood procaine level was reached, the height of which was proportional to the rate of infusion. The relation between in fusion rate and blood level of procaine is shown in Fig. 4, which summarizes the data set out in Fig. 3. The blood levels indicated in Fig. 4 are the equilibrium values.

Single injections of procaine

In one experiment single injections of procaine were given (each injection took 30 sec) and blood levels were determined $\frac{1}{2}$ min, $\frac{1}{2}$ min, $\frac{1}{2}$ min, and $\frac{1}{2}$ min after completion of the injection An interval of 30 min was allowed between each

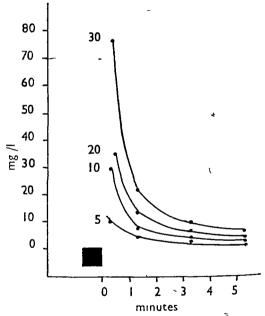


Fig 5—Blood procaine concentrations after single injections of procaine. In each curve the numeral at the top shows the dose in mg of procaine injected. The black square indicates the period of injection (4 min)

injection so that blood procaine levels could return to zero before the next injection. Fig. 5 shows the initial high blood procaine concentrations and the subsequent very rapid decreases after injections of 5, 10, 20, and 30 mg of procaine. The highest rate of fall is during the first minute after the end of the injection—e.g., after 30 mg procaine the blood level was 77 mg/l at ½ min and 22 mg/l at 1½ min after the end of the injection

Interpretation of blood level changes after single injections of procaine

It will be seen in Fig 5 that after a single injection of 30 mg procaine in 30 sec the blood procaine level fell from 77 mg/l to 75 mg/l. within 5 min of completion of the injection Reference to Fig 2 shows that after the termination of an infusion of procaine the blood level fell from 65 mg/l to 25 mg/l within the same period. The factors which produce this significant difference in rates of decay will now be considered.

After a single injection the fall in blood procaine level may be due to the following factors —

- 1 Diffusion from the blood stream into the tissue fluids and cells
 - 2 Fixation by tissues
- 3 Destruction in the blood stream, tissue fluids, and cells
 - 4 Excretion

It will be shown later that urinary excretion is negligible, and there is no evidence for significant elimination in any other way, so that the difference between the decay curve after a single injection and that after a continuous infusion must be related to the other factors. After a single injection lasting ½ min there must be a considerable element of diffusion from the blood into the tissues,

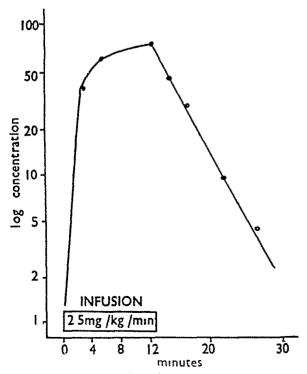


Fig 6—Ordinates blood procaine concentrations (from Fig 2) on logarithmic scale Abscissae time in min Note the straight line decay in procaine concentrations

whereas after the end of an infusion, when a plateau blood level has been reached, this factor would be negligible. Since the blood contains a higher concentration of the enzyme procaine esterase than any other tissue (Kisch, Koster, and Strauss, 1943) this blood enzyme would seem to be the most important factor in the fall in blood procame level after the end of an infusion were so there should be an exponential decay curve typical of such enzymatic processes, and Fig 6 shows that when the decay curve from Fig 2 is replotted with blood procaine concentrations on a logarithmic scale a straight line relationship is This observation, of course, does not locate the site of enzymatic destruction, whether in blood or tissues, but it does help to distinguish the factor of diffusion from that of destruction in the following way If it be assumed that 5 min after a single 1 min injection of procaine diffusion from the blood has virtually ceased, and further that even diffusion into the whole body water (intracellular and extracellular) has occurred, then it is possible to calculate what proportion of the decay curve is due to diffusion and what to enzymatic destruction

In the experiment recorded in Fig 5 the cat weighed 2.2 kg, and according to Gregersen (1941) the blood volume would therefore be 250 cc, the extracellular fluid volume 550 cc, and the total body water 1,500 c c If 30 mg procaine be evenly distributed throughout the total body water the blood level would be 20 mg/l and if diffusion were incomplete a higher value would be found The actual blood level 5 min after completion of the injection was 70 mg/l Similarly, the procame concentrations to be expected after 20, 10, and 5 mg injections would be 135, 68, and 34 mg /l respectively, the actual blood levels were 45, 20, and 09 mg/l It is therefore evident that in each case about two-thirds of the injected procame has been destroyed or fixed in the tissues in After injection of 10 mg of procaine the p-aminobenzoic acid level (2.5 mg/l) suggested that the disappearance of this fraction of procaine was due almost entirely to hydrolysis and not to tissue fixation From the data given by the decay curve in Fig 2 the reduction in blood procaine levels due to enzyme action can be estimated (see Appendix) and the reduction due to diffusion can be calculated by difference from the values given in Fig 5- In Fig 7 the continuous line shows the curve of enzymatic breakdown that would be expected if diffusion were instantaneous, and the dotted line the fall in blood level produced by diffusion alone

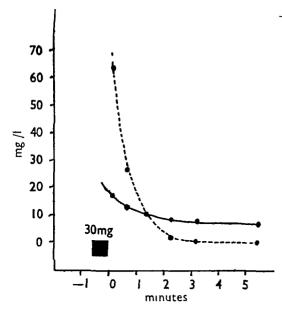


Fig 7—Curves showing contributions of diffusion (o---o) and enzymatic destruction (•---o) to fall in blood procaine concentrations after injection of 30 mg procaine in ½ min. The curves have been obtained by calculations described in the text and the Appendix, and should be compared with that after 30 mg procaine in Fig 5

The kidney and procaine blood levels

The possibility that the kidney might destroy or excrete infused procaine quickly enough to influence blood levels was studied

- 1 By determining blood procaine levels before and after ligation of the renal pedicle
- 2 By measuring procaine excretion in the urine

Fig 8 shows the results of an experiment in which an infusion of procaine at a rate of 1.2 mg /kg /min was given for 30 min In the first infusion a peak procaine level of 22 mg/l was recorded, the p-aminobenzoic acid level rose steadily and remained at 20 mg/l when the procame level had fallen to 25 mg/l. after the end of the infusion The renal pedicles were then ligatured tightly to cut off all blood flow through the kidney, 80 min after the end of the first infu-The infusion was then restarted at the same rate as before and continued for 30 min peak blood procaine level was 24 mg/l at the end of the infusion period The p-aminobenzoic acid level which was 175 mg/l at the beginning of the second infusion rose more steeply than on the first occasion and finally reached a value of 50 mg /1 at 30 min after the end of the infusion

The difference between the peak procaine values of 22 mg/l during the first infusion and 24 mg/l during the second infusion (after tying the renal pedicles) is very small and suggests that procaine is neither destroyed nor excreted to any significant degree by the kidney. The steeper rise in p-aminobenzoic acid levels after ligature of the renal pedicle suggests a significant excretion of this substance by the kidney.

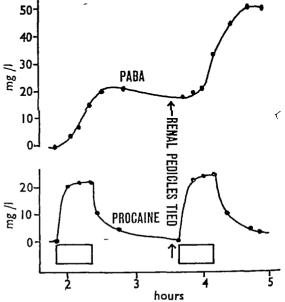


Fig. 8 —Effect of tying both renal pedicles on procaine and p-aminobenzoic acid blood concentration after infusions of procaine (1 2 mg/kg/min)

2 Urinary excretion of procaine and p-amino benzoic acid was studied in both cats and man In Fig 9 (b) the excretion of procaine and p-aminobenzoic acid in the urine of a cat is recorded for 2½ hr during and after an infusion of procaine at a rate of 0.8 mg/kg/min for 25 min. The blood level was steady at 21 mg/l 21 per cent of the injected procaine was excreted as such during the infusion and in the subsequent 2 hr a further 1.4 per cent was recovered. This rate of excretion could play no significant part in the fall in blood level after the infusion ended. The excretion of p-aminobenzoic acid was much greater.

In Fig 9 (a) the excretion of procaine and p-aminobenzoic acid in human urine is recorded during and after an infusion of 2 g of procaine n 70 min, urine being collected for a total period of 5½ hr Only 0 3 per cent of the injected procaine appeared in the urine during this period. The smaller proportion of procaine found in human urine may be related to the finding of Kisch et al

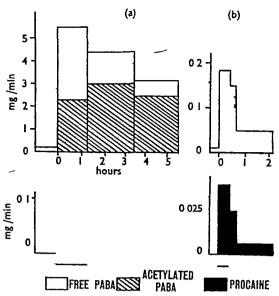


Fig 9—Urinary excretion of procaine, p-aminobenzoic acid (PABA), and acetylated PABA after procaine infusions in (a) a normal human subject and (b) a cat Ordinates amounts of the above compounds excreted in mg/min Abscissae time in hr The black horizontal lines indicate the periods of infusion (a) 2 g procaine in 70 min, (b) 0 8 mg/kg/min for 25 min

(1943) that the procaine esterase activity of human blood is greater than that of cat's blood. In human urine it will be noticed that a large proportion of p-aminobenzoic acid is acetylated, in cat's urine the acetylated fraction is negligible.*

The liver and procaine blood levels

The part played by the liver in the destruction of procaine was investigated by recording the effect of excluding the liver from the circulation on blood procaine levels during an infusion in an eviscerated cat.

In Fig 10 a procaine infusion of 0 65 mg/kg/min produced a plateau blood level of 14 mg/l Twenty min after the beginning of the infusion the hepatic artery was clipped and the infusion continued at the same rate. The blood procaine level rose rapidly to 24 mg/l and after 20 min of arterial occlusion the level was 25 mg/l, p-aminobenzoic acid blood levels rose steadily throughout the whole infusion period and there was no significant change in the slope of the curve during the period of hepatic artery occlusion

It was thought possible that the liver might be

concerned with the synthesis and liberation into the blood stream of procaine esterase, as has been claimed for cholinesterase by Brauer and Root The increased toxicity of procaine after liver damage noted by Ellinger and Hof (1929) might therefore be due to a reduction in blood procaine esterase To test this hypothesis liver damage was produced by intraperitoneal injection of carbon tetrachloride into rats and rabbits Each rat was given 0 075 c c of CCl, on five occasions at 2-day intervals The rabbits were given 04 cc/kg on the 1st day and 02 cc/kg on the 3rd and 5th days Serum procaine esterase determinations were made before and at intervals during the period of CCL administration, using the method described by Kisch and Strauss (1943) In rats the normal serum procaine esterase values were too small to give useful information, and the rabbits, in which high procaine esterase concentrations were found, showed no reduction with liver damage

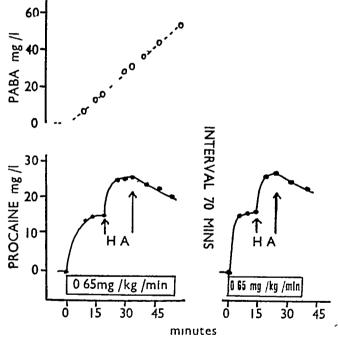


Fig 10—The effect of excluding the liver from the circulation during infusions of procaine. Upper scale p-aminobenzoic blood values. Lower scale procaine blood values. The experiment was done as described in the text in an eviscerated cat with the hepatic artery isolated and left patent. Between the arrows the hepatic artery was clipped.

Effects of physostigmine, neostigmine, methylene blue, and düsopropylfluorophosphonate on blood procaine and procaine esterase

Kisch (1943) reported a powerful inhibition of procaine esterase in vitro by physostigmine, neo-

^{*} Since this paper was submitted for publication Krebs Sykes and Barrley (1947) have found that in many animals including casts but not man there are enzymes which deacetylate p_{-} acetylatephonamides and which presumably could deacetylate p_{-} acetaminobenzoic acid The presence of such enzymes probably accounts for the very small amounts of p_{-} acetaminobenzoic acid found in the blood and urine of cats after procaine administration

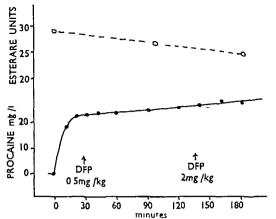


Fig 11—The effect of disopropylfluorophosphonate (DFP) on blood procaine and procaine esterase concentrations during a constant infusion of procaine at 1 0 mg /kg /min. The procaine esterase concentration is recorded above in arbitrary units. The procaine blood levels are recorded below. At the arrows DFP was injected.

stigmine, and methylene blue Doses of 1 mg physostigmine, 0.75 mg neostigmine, and 10-50 mg methylene blue had no effect on blood procaine levels during infusions into cats Dissopropyl-fluorophosphonate (DFP), in doses of 0.5 and 2 mg/kg, as shown in Fig 11 also produced no significant change in blood procaine level during an infusion of procaine at 1 mg/kg/min. The slight fall in serum procaine esterase activity and the slow small rise in blood procaine level noted in this record are probably due to the effects of repeated withdrawals of blood for determination of procaine concentrations

DISCUSSION

The administration of intravenous infusions of procaine, as described by Lundy (1942), Gordon (1943) McLachlin (1945), and Allen (1945, 1946), must be closely associated with the rate of inactivation of this compound in the body Rates of 10–50 mg procaine/min can be administered to human subjects for several hours with no evidence of cumulative action, and rates of up to 150 mg of procaine/min have been given for short periods, so that the processes of detoxication are extraordinarily rapid and efficient, this is also shown by the speed of recovery from the effects of intravenous procaine

The rates of infusion of procaine administered to cats correspond very closely to those given in man. In cats, as observed by Hill and Macdonald (1935) and confirmed by ourselves, rates in excess of 2 mg/kg/min are liable to produce respiratory failure, this would be equivalent to 12 c c of 1 per

cent procaine/min in a 60 kg man. It might however be expected that man would tolerate more than this by virtue of the greater procaine esterase activity of human blood, which is about 3 times that of cat's blood (Kisch et al., 1943)

It is clear from the results recorded in Fig 8 and Fig 9 that the kidney plays an insignificant part in the destruction or excretion of procaine, and the fact that in man less than 0.5 per cent of the injected procaine appears unchanged in the urine within a few hours shows that urinary excretion is of no importance

Since the marked tolerance to intravenous pro caine is not due to very rapid excretion, it may be assumed that the drug is speedily inactivated in the body 'Destruction of procaine by the liver was shown by Eggleston and Hatcher (1916) and by Dunlop (1935) and was postulated by Ellinger and Hof (1929), and destruction by blood in vitro has been demonstrated by Kisch et al (1943) and Hazard and Ravasse (1945) The question there fore arises as to how much the blood and liver respectively are responsible for the mactivation of procaine Our experiments on eviscerated animals give some idea of the proportion of procaine which may be destroyed by the liver The rate of pro came destruction bears an inverse relationship to the blood level (plateau level), the rate after exclusion of the liver is thus 14/24ths of the original rate, the liver having been responsible for the remainder of the destruction—viz, about 40 per cent of the total procaine catabolism

It was thought possible that the liver might secrete procaine esterase into the blood stream in a manner similar to that described for cholinesterase by Brauer and Root (1946), who showed that liver damage produced by carbon tetrachloride reduced the plasma cholinesterase level in rats, though sub sequently Ellis, Sanders, and Bodansky (1947) have found that this does not occur in rabbits experiments on serum procaine esterase levels in rats showed such low concentrations of this enzyme that the effects of liver damage could not be In rabbits, in which procaine esterase easily performed, determinations were damage caused no reduction in serum concentra-From this it is concluded tions of this enzyme that procaine esterase is not released from the liver into the general circulation in the rabbit.

Kisch et al (1943) found that physostigmine, neostigmine, and methylene blue all inhibited pro caine esterase in vitro. Neither these substances nor disopropylfluorophosphonate had any effect in full doses on the procaine blood levels in cats.

With slow infusions, once the initial diffusion

has occurred, the maintenance of a steady blood level is largely if not entirely the result of enzymatic destruction, and it is interesting to note that plateau blood levels are reached with all rates of infusion below those which depress respiration. In a cat weighing 3 kg , 5 mg of procaine can be inactivated per min , in a man weighing 60 kg this would correspond to 100 mg/min which has often been given with apparent safety

There seems to be little doubt that the destruction of procaine is in fact hydrolysis with the formation of p-aminobenzoic acid and diethylaminoethanol, the former of these two compounds having been frequently detected and estimated in blood and urine in the course of these experiments. The continued presence of fairly high p-aminobenzoic acid blood concentrations after the blood procaine levels have fallen almost to zero suggests that procaine which is bound to tissues immediately after diffusion is either slowly hydrolysed by such tissues or is liberated slowly from these tissues into the blood stream where hydrolysis takes place

We are very grateful to Mr W F Floyd for helpful criticisms of this work, and to Mr F J Haydon for technical assistance

SUMMARY

- 1 Blood procaine and p-aminobenzoic acid blood levels were determined in chloralosed cats after procaine infusions at rates of 0 5–2 5 mg/kg body wt/min, and after short single injections of 5–30 mg of procaine
- 2 With infusion rates of 0.5–2.5 mg/kg body wt/min, maximum blood procaine levels of 6–66 mg/l were recorded, and where the infusion was tolerated for at least 10 min constant blood levels were always attained Simultaneous p-aminobenzoic acid blood levels suggested rapid and extensive hydrolysis of procaine

- 3 Blood procaine decay curves were determined in six cats and found to be logarithmic
- 4 After short single injections of procaine the rapid fall in blood level during the first two minutes after the injection is due almost entirely to diffusion from the blood into the tissues
- 5 The kidney plays no significant part in the reduction of procaine blood levels during and after procaine infusions, but excretion of *p*-aminobenzoic acid is rapid and considerable
- 6 The liver is responsible for destruction of up to 40 per cent of the procaine metabolized in the cat.
- 7 There is no conclusive evidence that the liver "secretes" procaine esterase into the blood stream (rabbit)
- 8 Physostigmine, neostigmine, methylene blue, and DFP do not influence blood procaine levels during procaine infusion

APPENDIX

(Written with the help of Mr W F Floyd of the Department of Physiology, Middlesex Hospital Medical School)

Note on the calculation of enzymatic hydrolysis after rapid injection

When the type of substrate concentration-velocity curve for the enzyme is known the rate of enzymatic hydrolysis can be calculated from the mean rate of breakdown over the whole period of observation. After a single rapid injection the blood concentration is much higher than the tissue concentration during the first few minutes. Thus the assumption made here that the procaine is hydrolysed only in the blood will probably not involve a large error.

When the concentration of procaine in the blood

- 1	2	3	4	5
Time intervals min	C _t (mg /l)	C _t Km\delta(mg /l)	$\frac{W_0}{a} - \sum_{0}^{T} C_t K m \delta t$	Diffusion
0-0 25	95	2 66	17 34	77 66
0 25-0 75	64	3 55	13 79	50 21
0 75-1 25	36	2 00	11 79	24 21
1 25–1 75	26	1 45	10 34	14 66
1 75–2 25	11 8	0 65	9 69	2 11
2 25-2 75	9 6	0 56	9 13	0 47
2 75-3 25	8 4	0 47	8 66	-0 26
3 25-3 75	8 0	0 44	8.22	-0.22
3 75-4 25	7 6	0 42	7 80	-0.20
4 25-4 75	7 3	0 40	7 40	-0 10
4 75-5 25	7 0	0 39	7 01	0

is C_t , the concentration hydrolysed in the small time interval δt (C_t being the mid-interval concentration) is obtained by multiplying C by a coefficient proportional to the mean rate of breakdown occurring in unit time (i.e., the product Km) and by δt

Hence the total concentration hydrolysed in the time interval $O \rightarrow T$ is the sum of the concentrations hydrolysed in the successive time intervals δt . This is equal to the difference between the total amount injected (W_o) and that still unhydrolysed at time T (W_r) divided by the fluid volume (a) in which they are dispersed—

1e,
$$\sum_{t=0}^{T} C_t Km \, \delta t = (W_0 - W_T)/a$$

(Note the parameter K in the product Km is the velocity constant of the enzyme reaction, m is proportional to the enzyme concentration in the blood and for any one animal is a constant)

An example of the working for an injection of 30 mg of procaine into a 22 kg cat with a total body water of 151 is given in the Table

In order to calculate the entries for column 3 of the Table it is necessary to determine the value of Km as follows —

$$\sum_{t=0}^{5} \sum_{t=0}^{25} C_{t} = 233 \text{ (i.e., sum of total values of } C_{t})$$

$$W_{0} = 30 \text{ mg} \quad W_{T} = 105 \text{ mg} \quad a = 151$$

$$\delta t = 05 \text{ minutes}$$

$$Km = \frac{W_{0} - W_{T}}{a \delta t} = \frac{30 - 105}{15 \times 05 \times 233} = 01114$$

In Fig 7 the continuous curve is that given by column (4) and the dotted curve by column (5)

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EFFECTS OF BAL AND BAL GLUCOSIDE IN ACUTE LEAD ACETATE POISONING

BY

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(Received August 14 1947)

2 3-Dimercaptopropanol (BAL) was described by Peters, Stocken, and Thompson (1945) as an antidote to lewisite and other forms of arsenical poisoning. It has also been found to protect animals poisoned by several other heavy metals, but so far as is known the only data on its effect in lead poisoning are those of Braun, Lusky, and Calvery (1946), who found that after single or repeated intraperitoneal injections of lead nitrate into rabbits courses of BAL increased the mortality above that of control groups of animals. The experiments reported in the present paper are part of a study of the action of BAL and other dithiols in lead poisoning.

MATERIALS AND METHODS

Materials

The BAL used in this investigation was a waterpurified sample kindly presented by Prof R A Peters It was stored in a refrigerator and solutions were prepared freshly as required Solutions of BAL glucoside (Danielli, Danielli, Mitchell, Owen, and Shaw, 1946) were prepared from its barium salt by liberating the free dithiol with sulphuric acid, precipitating any residual barium with sodium sulphate and removing the precipitate by centrifuging strength of the solutions was estimated by iodine titration before use this gave somewhat variable results, but was more satisfactory than computing the strength from the amount of barrum salt used. as the latter varied considerably in its thiol con-The doses quoted must be regarded as only approximate

Red cell fragility experiments

Freshly prepared, heparinized, and washed rabbit red blood cells were allowed to react with lead acetate or BAL or plasma or mixtures of these, usually for one hour at room temperature. Their fragility was then determined by adding aliquots to a series of sodium chloride solutions ranging from 0.28 to 0.64 per cent (w/v) at intervals of 0.04 per cent, centrifuging and estimating the haemoglobin concentration in the supernatant fluid by visual comparison with

standards at 20 per cent intervals prepared from a water-laked suspension of the same red cells percentage lysis plotted against the salt concentration gave a sigmoid curve from which the 50 per cent lytic concentration (LyC50) was obtained graphically The probit of the percentage lysis plotted against the logarithm of the salt concentration allowed a straight line to be fitted fairly closely, such lines did not give a substantially different estimate of the LyC50 and were used only when a quantitative estimate of the slope was required For measurements in vivo the same procedure was followed, except that aliquots of the washed cell suspensions were added immediately and without other manipulation to the series of sodium chloride dilutions

Blood estimations

Reticulocyte and red cell counts were performed by the usual techniques Haematocrits were determined in capillary tubes of 0.1 ml capacity Haemoglobin was estimated in early experiments as carboxyhaemoglobin by means of a dilution comparator and later as cyanmethaemoglobin (King, Gilchrist, and Delory, 1944) by means of a Hilger Spekker absorptiometer calibrated with rabbit blood of known oxygen capacity Calculations based on duplicate estimations made in the course of these experiments gave the standard error of the red cell counts as $\pm 0.22 \times 10^4$ cells per cu mm and of the haemoglobin determinations by the cyanmethaemoglobin method as ± 0.28 g haemoglobin per 100 ml

Rabbit metabolism experiments

Adult rabbits of both sexes and various breeds were kept in metabolism cages for 22 hours a days and fed in the remaining two hours on bran, oats, greens, and turnips. Water was allowed ad libitum. Lead acetate was given by stomach tube and dithiols by injection into the muscles beside the vertebral column. Three to five rabbits were handled in a single experiment. Of these, one was treated with lead acetate and either olive oil as used to dissolve the BAL or glucose equivalent to the amount of BAL glucoside given to the other rabbits, sometimes one was treated with a dithiol and no lead, and the rest received lead and a dithiol. The rabbits were allotted to different treat-

ments by a random procedure Variation due to external conditions therefore affected all groups as far as possible equally

Coproporphyrin estimations

Coproporphyrin was estimated, usually in duplicate, on toluol-preserved three-day samples of urine and on three-day samples of faeces ground with anhydrous sodium sulphate, as described by O'Brien (1946) The urine and faeces were acidified with glacial acetic acid and extracted with ether. The ether was washed with 2 per cent (w/v) sodium acetate and extracted with 5 per cent HCl The HCl was neutralized with solid sodium acetate and extracted with ether, this ether was extracted with 0.5 per cent HCl The last three steps were repeated once or twice if necessary to give a bright red fluorescent solution The faecal extracts were taken into 0.2 per cent HCl and washed with chloroform before estimation Completeness of extraction was checked at each stage by observing the absence of fluorescence in ultra-violet light. The coproporphyrin in the extracts was estimated fluorimetrically in 05 per cent HCl against a standard solution of coproporphyrin I, kindly provided by Mr J R P O'Brien, in a Rimington-Schuster comparator (Rimington, 1943) The standard error, calculated from duplicate estimates, in these experiments was ± 8.2 per cent for urines containing 3 to 100 μ g per 100 ml The porphyrin was not further identified, but in view of the technique of extraction used and the well-established excretion of coproporphyrin in lead poisoning (Fischer and Duesberg, 1932, Watson, 1936, etc) there is little doubt as to the substance estimated

RESULTS

1 Preliminary experiments

Preliminary experiments were carried out in mice Promising results were obtained (Table I), but it was difficult to produce lead poisoning suitable for experimental study in these animals. They were very resistant to lead salts given by

TABLE I
The effect of BAL and BAL glucoside injected intramuscularly in mice poisoned by lead acetate injected
intraperitoneally

Dose of lead acetate mg /kg /day for 5 days	Dithiol	Dose of dithiol mg /kg /day for 5 days	Mor tality at 14 days from start of expt	differ contr	cance of rence, ol and ited
50 50 50	None BAL BAL glucoside	20 1000	10/10 7/10 3/10	1 57 7 90	about 0 2 <0 01

stomach tube or in the diet, and they suffered severely from the local necrotic action of solutions injected into the peritoneum or intra muscularly Larger and less resistant animals—viz, rabbits, were therefore used, in which haematological changes could conveniently be taken as an index of lead poisoning (Flury, 1934, for review) The experiments were based particularly on those of Aub, Reznikoff, and Smith (1924a and b) in vitro and of Key (1923) in vivo, as they appeared to provide means whereby toxic effects of lead could be produced rapidly and reproducibly

II In vitro experiments Fragility of normal rabbit washed red cells

The sigmoid curve relating the percentage lysis to the logarithm of the salt concentration was found to be fairly constant in shape but to vary somewhat in position Variation occurred from day to day in the same rabbit and between the mean values for different rabbits The former factor was the more conspicuous From determinations on 22 rabbits, the mean LyC50 was found to be 0.51 per cent with a standard deviation of the individual values of ± 0 042 per cent NaCl When the probit of the percentage lysis was plotted against the logarithm of the salt concentration, lines with a slope (b) of -20 to -30 were obtained

Fragility of red cells after exposure for one hour to lead acetate (9 µg Pb per ml)

After exposure to lead acetate, the cells were more resistant to lysis by hypotonic saline As was found by Aub, Reznikoff, and Smith (1924a), the change apparently affected mainly those cells which were in any case most resistant, as there was a much greater difference in the salt concen tration necessary to effect over 50 per cent lysis than in that to effect 10 or 20 per cent lysis Hence the fragility curves were flatter than normal, and the slopes of the transformed curves were in the region of -10 The reduction in the LyC50 varied (Table II) and was apparently The effect was not related to the initial value smaller if the fragility was estimated half an hour instead of one hour after exposure to lead acetate

Fragility of red cells after exposure for one hour to BAL

After exposure to 22.7 μg BAL per ml, the cells were slightly more resistant to hypotonic haemolysis than normal. In one or two experiments in which 113 μg BAL per ml were used,

TABLE II

The mean fragility of rabbit red blood cells exposed to lead acetate and BAL

F*				
Observation	No of obser- vations	Mean ± S E % NaCl	S D of indivi- dual values	P
Normal LyC50 - LyC50 after lead ace-	*	0 51 ±0 009	±0 042	
tate 9 µg Pb/ml LyC50 after BAL	51	0.35 ± 0.010	±0 068	
22 7 µg /ml LyC50 after lead ace-	5	0.48 ± 0.015	±0 034	
tate 9 µg Pb/ml + BAL 22 7 µg /ml Mean difference, nor-	5	0 48 ±0 017	±0 038	
mal and lead treated cells	51	0 15 ±0 011	±0 079	<0 001
Mean difference, nor- mal and BAL treated cells Mean difference, nor-	5	0 03 ±0 009	±0 020	<0 05
mal and BAL + lead treated cells	5	0 03 ±0 011	±0 024	<0 05

The 50 per cent lytic concentrations (LyC50) were determined graphically from sigmoid fragility curves

*Weighted mean of 54 observations on 22 rabbits

the change was more obvious. It was not accompanied by any appreciable change in the slope of the fragility curve

Fragility of red cells after exposure to lead acetate and BAL

When BAL was added immediately after lead acetate (9 µg Pb per ml) the effects of lead on the shape and position of the fragility curve were more or less prevented (Table II) 1 13 μg of BAL per ml (02 mols per mol of lead acetate) had no effect, $57 \mu g/ml$ (1 mol per mol) prevented about 80 per cent of the effect, and 227 μ g /ml (4 mols per mol) prevented it almost or quite completely The effect of lead acetate took some time to develop fully It was incomplete at half an hour, and if BAL was added at this time, further development was prevented but there was very little reversal of the established change. this was so even if BAL was allowed to act for three hours or if BAL concentrations up to 20 mols per mol of lead acetate were used

Fragility of red cells after exposure to lead acetate and BAL glucoside

Like BAL, solutions of BAL glucoside added immediately after lead acetate abolished or reduced the lead effect. Mol for mol the activity of the glucoside appeared to be of the same order as that of free BAL Influence of normal rabbit plasma on the lead acetate-red cell system

The results described above suggested that inhibition of the effect of lead acetate on red cells might provide a much needed method for assaying fairly small quantities of BAL in plasma fragility of normal red cells showed only small and variable changes when up to 18 per cent (v/v) of plasma was present in the reaction mixture, whether the plasma came from the same or a different rabbit. As was found by Aub, Reznikoff. and Smith (1924a) plasma inhibited the effect of lead, but this inhibition was fairly variable With 9, 135 or 18 per cent (v/v) of plasma, usually about two-thirds of the effect of lead acetate was prevented, but values from 7 to 100 per cent were sometimes obtained There was no obvious difference between the responses to plasma from the rabbit which had supplied the cells and those to plasma from other rabbits

Influence of normal rabbit plasma and BAL on the lead acetate-red cell system

When BAL and plasma were both added to the reaction mixture immediately after the lead acetate, there was always less inhibition of the lead effect than was produced by the similar amount of BAL alone. Sometimes there was also less inhibition than that produced by plasma alone, particularly with the higher concentration of plasma (18 per cent (v/v)) and smaller concentrations of BAL (57 μ g/ml) This smaller inhibition occurred also if the BAL and plasma were added an hour after the lead acetate, or if the BAL and plasma were mixed half an hour before addition to the reaction mixture of red cells and lead acetate

Influence of plasma from BAL treated rabbits on the lead acetate-red cell system

Two rabbits were used in each experiment. The first rabbit was bled (1 ml) and injected intramuscularly with 50 mg/kg BAL in nut oil or in 66 per cent (v/v) propylene glycol in water, or, in control experiments, with solvent alone Further 1 ml samples of blood were taken half, two and four and a half hours after injection The samples were heparinized and centrifuged and the plasma was separated at once The anti-lead activity of the plasma so obtained was assayed on lead acetate-red cell systems prepared about the same time with cells from the second rabbit. Sometimes the samples were re-assayed using other cells next The results are given in Table III. There was, in general, not an increase but a decrease in

TABLE III

ANTI-LEAD ACTIVITY OF PLASMA FROM RABBITS BEFORE AND AFTER INJECTION OF BAL

The 50 per cent lytic concentrations were determined graphically from sigmoid fragility curves Figures in the four final columns are the percentages of the lead effect prevented by adding 0.1 ml plasma to 1.0 ml of cell suspension treated with lead acetate,

1 e
$$\frac{\text{(Plasma + lead) LyC50-Lead LyC50}}{\text{Normal LyC50-Lead LyC50}} \times 100$$

Duplicate estimates on separate fragility systems are shown independently The final averages for BALtreated and untreated rabbits are based on the mean values for each rabbit.

Experiment			50% Lytic concentrations % of lead effect prevented by plasm									
Material		bbit Exp Nor- + + PbAc ₂ + Plasma										
ınjected	No	No	mal	PbAc ₂	Before injec	Hrs	after ii 2	пјес 4 1	Before injec	Hrs	after in	ijec 4 1
BAL 50 mg /kg in nut oil	43 43	4 <i>a</i> 4 <i>b</i>	0 63 0 48 0 48	0 29 0 39 0 35	0 52 0 46 0 46	0 30 0 46 0 46	0 43 0 42	0 46	70 78 85	3 78 85	 45 54	— 85
"	48 52	4 <i>d</i>	0 56 0 55 0 49	0 25 0 28 0 35	0 44 0 41 0 46	0 28 0.28 0 44	0 26	0 49	61 48 79	10 0 64	3	74
BAL 50 mg /kg in propy- lene glycol	49	4g 4h	0 49 0 51 0 48 0 47	0 30 0 29 0 34	0 43	0 36	0 40	0 45	70	40	48 77	71
Average values for BAL-tr	eated ra	bbits	II		l		ļ	L]	70	40	45	71
Nut oil	48	4 <i>f</i>	0 55 0 55 0 47	0 30 0 42 0 37	0 52	0 52	0 50	0 47 0 43	92 ~	92	69 °	38 50
"	51	4g	0 49 0 51	0 35	0 46	0 46	0 39	0 44	79	79 —	43	66
Propylene glycol	51	4/1	0 48 0 47	0 29 0 34	0 42	0 40	0 45	0 40	70 —	60 	85	46
Average values for contro	rabbits	 S	l 	1	J	I	 	I—	80	70	66	52

the anti-lead activity of plasma after BAL Much weight cannot be attached to this finding, because some fall occurred also in the later samples from the control rabbits however, large changes were observed only in the post-BAL plasmas (e.g., exp 4a, 4d) and especially in the half-hour sample Such a change is consistent with the purely in vitro finding that small amounts of BAL can diminish the anti-lead activity of plasma. In experiment 4d, the effect of the plasma samples on the fragility of cells without lead was examined. Neither the normal plasma nor the samples after BAL had any effect on the normal fragility. The effect of the plasma therefore seems to be on the lead rather than directly on the cells

Aub, Reznikoff, and Smith (1924b) claimed that the extent to which plasma or serum prevented the effect of lead on red cells was parallel to the phosphate concentration of the plasma or serum The phosphate concentration of four sets of plasma, two from control and two from BALtreated rabbits, were therefore estimated by a small Briggs-Bell-Doisy scale modification of the method, using a Hilger Spekker absorptiometer for the colour intensity determinations arose in that it was generally difficult to bleed rabbits by a single venepuncture when they had been treated with BAL, and that in samples obtained by allowing blood to drip from the ear some haemolysis occurred and the plasmas were pink or red These plasmas gave a high phosphate content, presumably owing to phosphate liberated Excluding samples with from damaged cells visible lysis, no change exceeding ± 5 per cent in phosphate concentration was found in control or BAL plasmas, although their anti-lead activity No correlation, positive or varied considerably negative, was observed between the presence of haemoglobin in plasma samples and the anti-lead activity

III In vivo experiments Changes in untreated lead acetate poisoned rabbits

The normal blood picture and coproporphyrin excretion of stock rabbits in this laboratory are summarized in Table IV After a single dose of

TABLE IV

Blood picture and coproporphyrin excretion of normal laboratory rabbits

		1		
	ਰੰ	Ş	Combined	
R B C × 10-6/cu.mm Haemoglobin g /100 ml Haematocrit % Reficulocytes per 100	14 3±1 7 41 5±0 3	59 ± 04 126 ± 17 384 ± 15	$135\pm18 \\ 398\pm12$	
R B C Mean corpuscular haemo- globin concentration,	_	17±13	14±10	
uμg /cell Mean corpuscular volume, cu μ	$\begin{bmatrix} 23 & 6 & \pm 3 & 3 \\ 66 & 7 & \pm 2 & 9 \end{bmatrix}$	215 ± 32 653 ± 17	$\begin{vmatrix} 22.7 \pm 3.2 \\ 65.9 \pm 2.3 \end{vmatrix}$	
Coproporphyrin excretion µg /day, urine faeces	4 1 ± 2 6 5 1 + 4 7	57±36	48±31	
,, 155665)	10120	X O I U	

The values given are the mean and standard deviation of individual values for nine male and seven female rabbits

300 mg/kg (08 mM/kg) of lead acetate by stomach tube, anaemia, reticulocytosis, punctate basophilia, slight albuminuria, haemoglobinuria, and increased coproporphyrin excretion were observed (Table V and Fig 1) The anaemia varied in severity. It was greatest about the third to sixth day, and recovery took three to six weeks The mean corpuscular haemoglobin concentration did not vary significantly, though apparent high values tended to occur at the onset and low values appeared in the second week The reticulocyte count rose rather slowly and reached its peak after about a week Punctate basophils were found roughly in proportion to the number of reticulocytes present The red cell fragility did not change as strikingly as in the rather similar experiments described by Aub, Reznikoff, and Smith (1924a) In the present experiments changes of fragility were observed mainly in rabbits which developed the worst anaemia In these the fragility curve became flatter, with slopes of about -12, and the position of the LyC50 showed little change If anything it increased, though by not more than 0 10 per cent NaCl, indicating that the cells were less resistant to hypotonic haemolysis The change in LyC50 did not exceed normal limits flattening was similar to that observed in vitro. Haemoglobinuria, somethough less definite times sufficient to produce a dark brown urine, occurred in the first two or three days, but no red cells were found in the urine. The excretion of coproporphyrin was greatly increased, mainly in the urine, and continued for at least nine days in one rabbit quantities above normal were still found after three weeks. Of the eight rabbits treated with lead acetate and no dithiol, three died. In

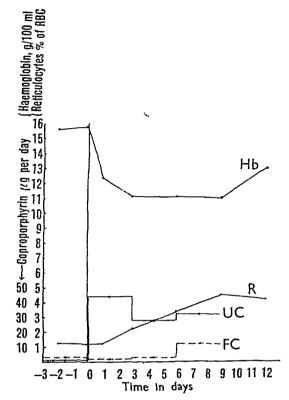


Fig 1—Acute lead acetate poisoning in rabbits Ordinates haemoglobin (Hb) g/100 ml, reticulocyte count (R) per 100 R.B.C., coproporphyrin excretion μg/day, UC, urinary, FC, faecal Abscissae, time in days from administration of lead acetate, 300 mg/kg, by stomach tube Values for haemoglobin and reticulocytes based on eight rabbits and for coproporphyrin excretion on three rabbits

one of these the haemoglobin fell by over half in the first day and its death on the second day was probably due to anaemia. The other two died on the third and sixteenth days respectively, without gross anaemia or other obvious cause

Changes in rabbits receiving only dithiols

The actions of dithiols alone are being studied further and will be reported in detail separately. The amount of BAL used in the present experiments caused transient haemoconcentration and occasionally a rise in reticulocyte count. Otherwise the blood picture and red cell fragility were unaltered. Increased excretion of coproporphyrin, or of a red fluorescent pigment which behaves

TABLE V

INFLUENCE OF DITHIOLS ON THE EFFECTS OF LEAD ACETATE IN RABBITS

The fall in R.B.C. haemoglobin and haematocrit are calculated from the mean difference between two control values before treatment and values on the first and third days after administering lead acetate The values given are the mean and standard error for all the rabbits in each group The coproporphyrin excretion figures are those for the first three days after administration of lead acetate, and are based on 2 or 3 rabbits in each group

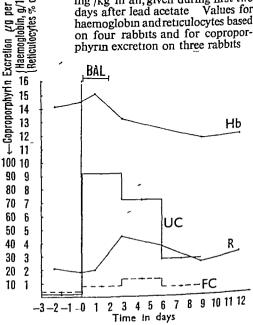
Lead acetate 300 mg/kg orally and dithiols intramuscularly	No of rab- bits	No died	Fall in RBC × 10-6	Fall in Haemo- globin g /100 ml	Fall in h'crit %	Haemo- globin- uria	phyr.	opor- in ex- tion day Fc	Signifi of diffi fro untre R.B C	erence om eated
No dithiol BAL 50 mg/kg at 1 hr +	8	3	1 64±0 41	4 23±1 07	12 0	++	45	13	_	
25 mg/kg 8-hrly for 7 doses BAL glucoside 250 mg/kg at 1 hr + 125 mg/kg	4	2	0 12±0 19	0 19±0 63	22	Trace	92	94	13 55	0 01
8-hrly for 7 doses BAL glucoside 250 mg/kg	3	0	1.23±0 44	2 25±0 46	11 5	0 to +	50	14 0	2 16	01
at 1 and 5 hours BAL 25 mg/kg + BAL glucoside 125 mg/kg at 1 hr + BAL 12 5 mg/kg	4	0	0 47±0 22	1 02±0 37	4 0	0 to +	- 1	_	9 85	0 01
+ BAL glucoside 62 5 mg / kg 8-hrly for 7 doses BAL glucoside 250 mg /kg	2	0	0 52	1 30		0 to +	42	98		
at 21 hrs + 125 mg/kg 8-hrly for 4 doses	1	0	2 60	6 85	17 5	++	_			-,

similarly, occurred consistently Assuming the pigment to be coproporphyrin, the excretion after doses of BAL such as were used here was of the order of 12 to 20 µg/day BAL glucoside alone had no appreciable effect on the blood picture Its effect on porphyrin excretion has not been studied

Effect of dithiols in lead acetate poisoned rabbits

Lead poisoned rabbits treated with BAL in a dosage of 50 mg/kg (0 4 mM/kg) intramuscularly one hour after the lead acetate followed by 25 mg/kg every eight hours for two days-1e, to a total dosage of 200 mg/kg (16 mM/kg) developed very little anaemia as long as BAL was being administered Later the anaemia increased. but the fall was less than in untreated animals (Fig 2) Reticulocytosis was smaller, and occurred earlier than in rabbits receiving lead acetate but no BAL This response was somewhat variable and the difference may be due to chance coproporphyrin excretion was greater than in rabbits receiving no dithiol, but subsequently fell off more quickly Although there was good protection against the anaemia, the mortality vias not appreciably altered, as two rabbits of the four in

Fig 2-Acute lead acetate poisoning treated with BAL Ordinates and abscissae as in Fig 1 BAL, 200 mg /kg in all, given during first two days after lead acetate Values for haemoglobin and reticulocytes based on four rabbits and for coproporphyrin excretion on three rabbits



this group died, on the fifth and twelfth days respectively

Treatment with BAL glucoside (Fig 3) on a similar dosage system but with a total dosage of about 1,000 mg/kg (3 5 mM/kg) had only a slight and not significant effect on the anaemia However, when the BAL glucoside was given in two 250 mg/kg. doses one and five hours after the lead acetate, it was about as effective as BAL

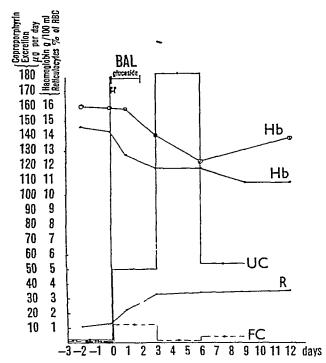


FIG 3—Acute lead acetate poisoning treated with BAL glucoside Ordinates and abscissae as in Fig 1 BAL glucoside, 1,000 mg/kg in all, given during first two days after lead acetate Values for haemoglobin and reticulocytes based on three rabbits and for coproporphyrin excretion on two rabbits. The line joining points enclosed by circles is for a group of four rabbits treated with BAL glucoside, 500 mg/kg in all, given during the first six hours after lead acetate.

in delaying the onset of anaemia Porphyrin excretion and reticulocytes were not followed in the latter group In the former, as in BAL treated rabbits, the coproporphyrinuria was greater and the reticulocytosis less than in those receiving lead acetate alone Of a pair of rabbits treated with BAL and BAL glucoside, as suggested by Danielli et al (1946), one developed very little and one considerable anaemia Both produced large amounts of coproporphyrin One rabbit treated with BAL glucoside twenty-one hours after lead acetate, by which time anaemia was well developed, did not become more anaemic, but did not recover more rapidly than those of the control group In all the dithiol treated rabbits, red cell fragility changes occurred as in the controls—ie, only when there was fairly severe anaemia None of the eight rabbits treated with BAL glucoside alone died, nor did the two treated with BAL and BAL glucoside together

DISCUSSION

In suitable concentrations BAL prevented the action of lead acetate on rabbit red blood cells If BAL was added half or one hour after the lead acetate, the effect of BAL was small and consisted chiefly in preventing the full development of the effect of the lead, not of significantly reversing an established change in fragility rate of uptake of lead ions by red cells in vitro is fairly slow (Behrens and Pachur, 1927, Mortensen and Kellogg, 1944) in concentrations of the order of those used here, and, to judge from Mortensen and Kellogg's data, corresponds quite closely with the development of the fragility The action of BAL therefore appears to be one of mactivating lead ions not yet taken up by cells, rather than of actual de-leading cells or altering the cell lead so as to prevent its fragility effect There is certainly no reversal of poisoning comparable to that which occurs with arsenicals -eg, of pyruvate oxidase by lewisite (Stocken Further experiments are and Thompson, 1946) being conducted to see what effect BAL has on the uptake of lead by red cells in vitro and in vivo

Plasma, like BAL, prevented the effect of lead However, the combination of acetate in vitro BAL and plasma, particularly in the proportion of 30-60 µg BAL per ml of plasma, was less effective than either alone, and a similar reduction in plasma anti-lead activity occurred in vivo after the injection of large doses of BAL How this happens is not known It has been suggested that lead is taken up by plasma as inorganic phosphate (Aub and Reznikoff, 1924, Brooks, 1927), or as a double phosphate of lead and calcium (Bischoff and Maxwell, 1928, Maxwell and Bischoff, 1929, Jowett, 1932), or as an organic complex, probably with albumin (Teisinger, 1935) or citrate (Kety, In the present experiments the altered plasma anti-lead activity, which presumably reflects the capacity of the plasma to combine with lead, was not related to changes in inorganic phosphate Beyond that the mechanism has not been content studied

Interpretation of how BAL acts in vivo depends on the view taken of the mechanism of the lead anaemia (Cantarow and Trumper, 1944, Flury, 1934) In the present experiments the initial anaemia was clearly haemolytic, because of the rapidity of the fall and the appearance of haemoglobin in the urine The tendency of the mean corpuscular haemoglobin concentration towards high values at this stage was probably an artefact due to the presence of haemoglobin in the plasma The present observation that changes in fragility were most definite in rabbits with the severest anaemia is consistent with Aub, Fairhall, Minot, and Reznikoff's hypothesis (1925) that the primary change is in the red cell It does not follow that the anaemia of chronic plumbism has the same origin, and, in fact, the known changes in the bone marrow and the increased porphyrin excretion cannot be accounted for on this basis (Duesberg, 1931, Flury, 1934) particularly as there is no evidence of abnormal conversion of circulating haemoglobin to coproporphyrin (Bjorkmann, 1941, Kark and Meiklejohn, 1941) As increased coproporphyrin excretion occurred in the present experiments disturbances of blood formation as well as blood destruction were probably involved

In these circumstances the effects of BAL are unlikely to be simple Clearly BAL prevents the acute haemolytic anaemia as long as BAL is available in the circulation This is comparable with its action in vitro and may be similarly attributed to prevention of the uptake of lead ions by red Experiments now in progress on the effect of BAL on lead distribution in vivo have lent support to this hypothesis On the other hand, certain features of lead poisoning are enhanced, notably the coproporphyrin excretion and possibly the speed of the reticulocyte response Both these effects can be produced by BAL alone, but the coproporphyrin output is considerably more than can be accounted for by simple addition of BAL and lead effects

The actions of BAL glucoside are on the whole similar, though the inefficacy of the glucoside given in a course comparable with that used in the BAL experiments is not immediately explicable. Possibly this very water-soluble substance is excreted very rapidly. As the *in vitro* experiments with BAL suggest that the protection of red cells is mainly prophylactic, a sustained high concentration of thiol at an early stage would be important, and the shorter and more intensive course would be more likely to be effective

The number of rabbits used was too small for the mortality figures to be significant, though it is worth noting that all the BAL glucoside treated rabbits survived BAL treatment apparently did not save life, but there was no evidence that it increased the mortality as might have been expected, since the doses used were at the upper

limit of tolerance and those of lead acetate were within lethal limits These experiments differed from those of Braun et al (1946) in that BAL was given in a shorter and more intensive course at an earlier stage of poisoning The only observation made here which might relate to the increased mortality described by Braun et al is that of the diminished plasma anti-lead activity Assuming that this reflects a diminished affinity of the plasma for lead, it may account for larger amounts of lead being free to act at some more susceptible But there is no evidence as to what this site may be, or whether the effect has any relevance at all to the combined actions of lead and BAL Unfortunately it is not known whether BAL glucoside has a similar action on the plasma Experiments on this question have been prevented by lack of active preparations of the glucoside The action of BAL in preventing the effects of acute intoxication with lead, and perhaps in pro ducing some other fatal effect, and the prevention of poisoning by BAL glucoside are strongly reminiscent of their respective actions in cadmium poisoning (Gilman, Philips, Allen, and Koelle, 1946) and suggest further work on such lines The actions of BAL in chronic lead poisoning and with reference to the distribution of lead in the organism require further study The available evi dence does not warrant the use of BAL in clinical plumbism, but it would perhaps be premature to reject all dithiols as useless or dangerous It is also possible that they will throw further light on the still poorly understood mechanisms of lead poisoning

SUMMARY

- 1 In mice poisoned by repeated intraperitoneal injections of lead acetate the mortality was reduced slightly by BAL and significantly by BAL glucoside
- 2 In vitro BAL and BAL glucoside prevented the decrease in the fragility of washed erythrocytes due to lead acetate If the lead was added much before the thiol, the effect of the lead was scarcely at all reversed
- 3 Mixtures of BAL and plasma in certain proportions, and plasma from rabbits injected with BAL, protected washed erythrocytes from the effect of lead acetate less than did equal amounts of BAL or plasma alone
- 4 In rabbits poisoned by a single dose of lead acetate given by stomach tube BAL and BAL glucoside each significantly decreased the subsequent anaemia and increased the coproporphyrinuria. The mortality was apparently unaffected by BAL, but was reduced by BAL glucoside the

number of rabbits was too small for this difference in mortality to be significant

5 The significance of these findings is discussed

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STUDIES IN THE CHEMOTHERAPY OF TUBERCULOSIS PART I. SULPHONES

BY

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(Received August 25 1947)

After the introduction of prontosil and sulphanilamide for the treatment of streptococcal infections, much interest was taken in the use of sulphonamides and 4 4'-diaminodiphenylsulphone for the treatment of tuberculosis in guinea-pigs example, Rich and Follis (1938) showed that sulphanilamide exerted a striking effect (as judged by such criteria as spleen size, and distribution and extent of lesions) on such an infection, but it was not until four years later that Feldman, Hinshaw, and Moses (1942) using the N N'-bis-sodium dextrosebisulphite derivative of 4 4'-diaminodiphenylsulphone (promin) were able to demonstrate a definite difference in survival times between groups of treated and control animals We had begun the work reported here on sulphones as possible antituberculous drugs in 1942, and when the paper by Feldman et al appeared we were encouraged to proceed further along these lines, But it seemed to us unlikely that improved drugs would be found among solubilized forms of 4 4'-diaminodiphenylsulphone of the type represented by promin and diasone (which in all probability owe their activity to breakdown in vivo to the parent sulphone, with its known risk of serious toxicity), and it was our intention to examine sulphones of as widely differing chemical types as possible No rapid and simple screening test for selecting compounds active in vivo was available when the work started, and we were forced to rely upon an in vitro method, although the limitations of this approach were fully realized at the time To anticipate the main conclusion of the present report, it may be said at once that when a convenient in vivo method became available (Martin, 1946) the determination of in vitro activity was shown to be a completely unreliable guide for the discovery of compounds with activity in

vivo This conclusion is in accordance with current opinion among other workers (see, eg, Feldman and Hinshaw, 1945)

EXPERIMENTAL METHODS

In vitro—Each compound was finely ground with a small quantity of "Dispersol OG" and diluted with water to give a final concentration of drug of 1 100 Further dilutions (1 300, 1 900, etc.) were prepared from this and 05 ml of each dispersion added to 45 ml amounts of Long's synthetic medium containing 15 per cent of agar, to give final concentrations of compound as follows

Serial number of tube 1, 2, 3, etc Final concentration of compound 1 1,000, 1 3,000, 1 9,000

The medium was allowed to solidify with the tubes slightly inclined, and each tube was sown with a small particle of a culture of the H37 strain of "human" tubercle bacıllı on Lowenstein's medium Care was taken that all particles were as nearly as possible of the same size Tubes were incubated in a moist atmosphere for 14 days at 37°C and the degree of growth assessed by comparison with that in control tubes The in vitro activities of the compounds in all the following tables are quoted as 'in vitro indices" which were arrived at as follows The serial number of the last tube in which no growth took place is recorded as the first figure of the index, the second figure of the index is the serial number of the first tube in which full growth occurred index "4/6" would therefore indicate that the com pound in question completely inhibited growth (under the conditions described) in the fourth tube (ie, at a concentration of 1 27,000) and had no inhibitory action whatever in the sixth tube (i.e, at a concentra-The majority of the compounds tion of 1 243,000) listed in the following tables were tested at least twice and the indices found to be reproducible Similar limiting concentrations for complete inhibition of growth and complete absence of inhibition were found for a number of the more active compounds

using Long's liquid synthetic medium in which the organism was allowed to grow as a pellicle

In vivo —Therapeutic tests were carried out on groups, each of 24 mice, infected intravenously with 1 mg. of "human" tubercle bacıllı (strain 905) Details of the method have been published (Martin, The examination of each substance was preceded by a chronic toxicity test extending over a period of three weeks. The mice were selected and randomized as described for the therapeutic test Each compound was given at a range of doses using 12 animals for each dose level, and each group was weighed at weekly intervals The dose chosen for the therapeutic test was the highest which permitted normal growth, and on which the animals appeared to be in good condition. It was not always possible to choose this dose with full confidence, and in such cases one or more smaller doses were also given Doses are quoted in the tables as mg per 20 g mouse, and were administered orally twice daily for five days and once on Saturdays The first dose was given shortly before infection and dosing continued until the first specific death occurred in the control group— 1e, for about 14 days in most cases In the tables of therapeutic results the column headed "Increased mean survival time" gives the difference between the mean survival times of treated mice and untreated control animals The column headed "Increase required for significance" gives the time increase which would be necessary for statistical significance at the level P=0.05 If the figure in the first of these columns is positive and exceeds the value in the second column, the compound shows in vivo activity Negative values equal to or greater than the "required increase" indicate that the toxicity of the drug has had a significantly adverse effect on treated mice Values of magnitude approaching that required for significance (positive or negative) may be expected to arise through the operation of small uncontrolled variables once in twenty times, although the compound has no influence on the infection test will detect activity in vivo is shown by the results for 4 4'-diaminodiphenylsulphone (No 371) and 2 4'diamino - 5 - thiazylphenylsulphone (No 4879) (see Table XXIII)

Blood level concentrations—Only those compounds carrying aromatic amino groups were estimated. Details of the method used in these laboratories, for the determination of blood concentrations after the oral administration of such compounds, have been described previously (Martin, Rose, and Bevan, 1943). The method is based upon diazotization and coupling to form an azo colour.

RESULTS

It had been observed that the activity in vitro of diphenylsulphone itself (index 1/5) was not greatly different from that of 4 4'-diaminodiphenylsulphone (index 2/5). It was therefore permissible for the purpose in hand to disregard the

substituent amino groups of diaminodiphenylsulphone and attempt to find some combination of hydrocarbon residues which, united by the sulphone linkage, would possess higher intrinsic activity It was, of course, realized that such a compound would be unlikely as such to be effective in vivo and the next step envisaged was the introduction of groups such as amino, methoxy, etc. which it was thought might confer appropriate pharmacological properties on the new parent sulphone structure In Table II below are listed sulphones representing the various combinations of the groups phenyl, p-tolyl, 4-diphenylyl, α - and β -naphthyl, cyclo-hexyl, and cyclo-pentyl Highest activity seems here to be associated with hydrogenated cyclical nuclei, and further sulphones containing cyclo-alkyl and alkyl residues were tested. These are listed in Tables III, IV, V, and VI The high indices shown by phenyl-, p-tolyl-, and p-n-butylphenyl-alkylsulphones (containing alkyl residues with 5 to 8 carbon atoms) seemed sufficiently marked to warrant the undertaking of the second part of the investigation, namely the introduction of further substituents into selected parent sulphones From the Tables VII, VIII, X, XI, and XII it will be seen that the introduction of a single amino, alkoxy, or hydroxy group into the aromatic ring of such sulphones does not alter the in vitro index The β -diethylaminoethylamino group, however, reduces in vitro activity (Table IX), as does the presence of two methoxy groups (Table The presence of amino groups in some of these compounds made it possible to estimate them readily and the concentrations which were attained in the blood of mice after oral administration of

TABLE I

Blood concentrations attained after oral administration of maximum tolerated doses of various *m*-amino-*p*-tolylalkylsulphones

Num- ber	R	Dose mg /20 g mouse	(mg/10	concent 00 ml) a fter dosu 2 hr	at time
3631 3630 3622 2972	methyl n-propyl n-amyl n-heptyl	5 5 5 7 5	80 55 21	8 2 3 2 1 6	4 0 1 6 1 0

maximum tolerated doses were measured. Those compounds which have small alkyl groups were well absorbed, though somewhat rapidly excreted. With increasing size of alkyl group, the maximum

TABLE II

(a) Phenylsulphones SO_2R

Number	R	In vitro index
1645	phenyl	1/5
2651	<i>p</i> -tolyl	<1/6
2653	4-diphenylyl	1/3
2692	α-naphthyl	<1/6
2693	β-naphthyl	< 1/4
2763	cyclopentyl	2/4
2671	cyclohexyl	3/6

(h) p-Tolylsulphones CH ₃ SO ₂ R			
2599 2601 2624 2625 2603 2602 3113			

(c) 1.2.1pm	eny iy istripriones &)
2600 2626 2620 2627 2617	4-diphenylyl α-naphthyl β-naphthyl cyclopentyl cyclohexyl	<1/4 <1/5 <1/1 1/6 <1/5

(c) 4-DinhenvlylenInhones

(d) α- and β-Naphthylsulphones SO ₂ R			
2628 2618 2621 2619	(α) cyclopentyl (α) cyclohexyl (β) cyclopentyl (β) cyclohexyl	2/>6 3/>6 3/8 3/>6	

(e) D1-c) cloalkylsulphones SO ₂ R				
2747	cyclopentyl	2/5		
2723	cyclohexyl	2/6		

concentration reached in the blood decreased rapidly. This is illustrated for the *m*-amino-ptolylalkylsulphones (Table I)

While the work described above was in progress. attempts were also being made to obtain compounds more effective than 4 4'-diaminodiphenylsulphone by replacing the amino groups by other substituents Attention was concentrated on these compounds (Tables XIV and XV) rather than on phenylalkylsulphones when it was realized that members of the aminophenylalkylsulphone series having an alkyl group large enough to confer high activity in vitro were so poorly absorbed that high activity in vivo was most unlikely development of this aspect was the preparation of heteroarylphenylsulphones (Table XVI), interest in which was first aroused by reports by Feldman, Hinshaw, and Mann (1944) on the activity of promizole (2 4'-diamono-5-thiazylphenylsulphone, corresponding to our compound No 4879) against a tuberculous infection in guinea-pigs As a final variation the effect of replacing the sulphone linkage itself by other related linkages was examined The linkages chosen were the sulphonic ester (-SO₂O₂) and sulphonamide (-SO₂NH₂) groupings and compounds corresponding to both phenylalkyland diphenylsulphones examined (Tables XVII to XXI) Several of the phenylalkane and phenylbenzene sulphonates have very high in vitro indices, while those of the N-alkyl- and N-phenyl- benzenesulphonamides are about the same as those of the corresponding sulphones

Number	R	In vitro index
2892 2891 2760 2758 2845 2846 2842 2849 2952 2839 2843 2860 2672 2844	methyl ethyl n-propyl n-propyl n-butyl l-methylpropyl n-amyl isoamyl 2-methylbutyl n-hexyl 4-methylamyl n-heptyl n-octyl	<1/3 <1/4 1/4 1/4 1/4 3/>6 2/5 2/6 4/7 3/6 3/6 5/9 5/>8 4/9 5/8

TABLE IV

(a) p-Tolylalkylsulphones CH ₃ &	SO ₂ R
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Number	R	In vitro index
2988 2759 2764 3001 2650 2673	methyl n-propyl sopropyl n-amyl n-heptyl n-dodecyl	1/5 2/5 1/4 5/8 5/>6 <1/3

(b) p - n -Butylphenylalkylsulphones n - C_4H_9 SO ₂				
3403 3404 3405 3817 3406	methyl n-propyl n-amyl soamyl n-heptyl	2/8 5/>8 6/>8 5/>8 5/>8 2/8		

TABLE V $$\alpha$- and β-Naphthylalkylsulphones $$-SO_2R$$

Number	R	In vitro index
2840 2654 2652	(α) n-propyl(σ) n-heptyl(β) n-heptyl	3/>6 2/>6 1/6

TABLE VI Dialkylsulphones R-SO₂-R'

Number	R	R'	In vitro index
2858	c) clopentyl	n-heptyl n-propyl n-heptyl n-dodecyl n-heptyl	4/>8
2762	c) clohexyl		2/5
2722	cyclohexyl		4/>6
2761	c) clohexyl		<1/1
2942	n-heptyl		<1'>6

TABLE VII

p-Aminophenylalky lsulphones	NH ₂	SO₂R
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Number	R	In vitro index
3134	methyl	<1/3
3373	n-propyl	1/5
5505	<i>iso</i> propyl	1/4
1080	n-amyl	2/6
3808	isoamyl	1/4
3914	2-methy lbuty l	2/5
3910	ci clopenty l	1/4
3082	n-heptyl	4'6

TABLE VIII

<i>p</i> -Alkyl- <i>m</i> -aminophenylalkylsulphones R NH	⇒′
---	----

Number	R	R'	In vitro index
3631 3630 3622 2972 3558 3664 3889	methyl methyl methyl methyl methyl n-butyl n-butyl n-butyl	methyl n-propyl n-amyl n-heptyl n-propyl n-amyl soamyl	<1/5 2/5 5/8 4/7 4/>8 4/8 5/8

TABLE IX

p-β-Diethylaminoethylaminophenylalkylsulphones

$(C_2H_5)_2N(CH_2)_2NH$	$\int SO_2R$
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Number	'R	In vitro index
3160 3114 3110	methyl n-amyl n-heptyl	<1/3 <1/3 2/5

TABLE X
CH₃O
SO₂R

Number	Position of methoxyl group	. R	In vitro index
3717 3740 3458 3718 3739 3459 3721 3804 3479 3938 3722 3209	0 m p o m p o m p o	methyl methyl methyl n-propyl n-propyl n-amyl n-amyl n-amyl n-heptyl n-heptyl	1/4 <1/5 <1/4 1/4 2/5 3/7 3/6 4/8 5/>8 4/8 4/7 4/8

TABLE XI

	p-Alkoxyph	p-Alkoxyphenylalkylsulphones RO SO ₂ R'			
	Number	R	R'	In vitro index	
•	3465 3486 3520 3522	n-propyl n-propyl n-propyl n-amyl	methyl n-propyl n-amyl n-amyl	3,7 4>8 4>8 4>8 4>8	

TABLE XII



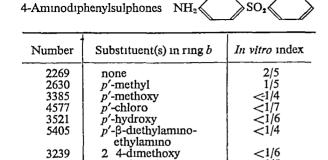
	Number	Position of hydroxyl group	R	In vitro
•	3460 3478 3745 3805 3494	p p o m p	methyl n-propyl n-amyl n-amyl n-amyl	1/4 1/5 4/6 4/8 4/7

TABLE XIII



	Number	Positions of methoxyl groups	R	In vitro index
_	4031 4032 4092 4093 3896 3895	2 4 2 5 2 4 2 5 2 4 2 5	methyl methyl n-propyl n-propyl n-amyl n-amyl	<1/3 <1/3 1/3 1/3 1/5 2/6 1/5

TABLE XIV



5-dimethoxy 4-dihydroxy

5-dihydroxy

3 5-dihydroxy

p'-amino

3624

4286 2790

3686

371

TABLE XV



Num- ber	Substituent(s) in ring a	Substituent in ring b	In vitro ındex
3384 3535 4326 3336 3236 3491 3335 3402 3537 3441 3551 3433	o-amino o-amino 3 4-dimethoxy p-methoxy 2 4-dimethoxy 2 4-dimethoxy 2 4-dihydroxy 2 4-dihydroxy 2 4-dihydroxy 2 4-dihydroxy 2 4-dihydroxy 2 4-dihydroxy p-chloro	p'-methoxy p'-hydroxy none p'-methoxy none p'-methoxy p'-hydroxy none p'-hydroxy none p'-hydroxy p'-chlòro p'-chlòro p'-chlòro	<1/4 -1/6 1/3 <1/4 <1/5 <1/5 3/6 3/6 3/6 3/5 <1/4 3/8 <1/5

TABLE XVI Heteroarylphenylsulphones R-SO₂R'

Num- ber	R	R'	In vitro index
2719 2745 2695 2694 4879 5163 5106	8-quinolyl 6-quinolyl 8-quinolyl 6-quinolyl 5-(2-aminothiazyl) 5-(2-amino-4- methylthiazyl) 5-(2-amino-4- methylthiazyl)	phenyl phenyl p-tolyl p-tolyl p-aminophenyl p-tolyl p-aminophenyl p-tolyl p-aminophenyl	<1/1 3/6 <1/4 <1/6 1/2 <1/2 2/5 3/6

TABLE XVII
Phenylalkanesulphonates R-SO₂OR'

Number	R	_ R′	In vitro
4033 4161 3723 3744 3737 3783 3983 4088	methyl methyl n-amyl n-amyl n-amyl n-amyl isoamyl isoamyl	p-anisyl p-hydroxyphenyl m-anisyl p-anisyl m-hydroxyphenyl p-hydroxyphenyl p-hydroxyphenyl p-aminophenyl	1/6 1/5 4/>8 5/>8 5/>8 5/>8 5/>8 4/>8 4/8

TABLE XVIII

Phenyl sulphanilates p-Aminophenyl benzenesulphonates SO_2O

			
Number	Substituent in ring a	Substituent in ring b	In vitro ındex
3898 3997 4089 3998 3616 4409 4590 4587 4090 4315 4144 4197	p-amino p-amino p-amino p-amino p-amino p-amino p-amino p-amino m-amino o-amino p-methoxy p-chloro	none o'-amino m'-amino p'-amino p'-methoxyl m'-hydroxy p'-hydroxy p'-chloro p'-amino p'-amino p'-amino p'-amino	2/5 2/5 <1/4 <1/5 <1/3 4/6 3/5 4/6 <1/5 3/5 <1/4 6/8

TABLE XIX
Miscellaneous Phenyl benzenesulphonates

а	\boldsymbol{b}
SO	20

Number	Substituent in ring a	Substituent in ring b	In vitro
4211 3233 4091 4314 3617 3899 4099 4142 4143 3900 3901	none none m-amino o-amino m-amino m-amino m-amino p-methoxy p-methoxy p-methoxy p-chloro	m'-methoxy o'-hydroxy o'-amino o'-amino o'-methoxy m'-methoxy p'-methoxy o'-amino m'-amino m'-methoxy m'-methoxy	4/7 4/6 <1/5 3/7 1/6 3/7 3/6 <1/7 3/5 4/6 5/6

TABLE XX N-Alkylbenzenesulphonamides R SO₂N \mathbb{R}'

Num- ber	R	R'	R"	In vitro index
2831 2985 2984 2829 2827 2990 2836 2992 4682 4735 3241 4730	methyl methyl methyl methyl methyl methyl methyl methyl amino amino amino	n-butyl n-amyl n-heptyl ethyl pentameth n-amyl n-butyl n-heptyl diethylaminoethyl amyl diethylaminoethyl	H H H ethyl ylene methyl n-propyl methyl ethyl ethyl ethyl	3/6 4/7 5/6 3/6 2/>6 4/7 4/7 4/6 <1/2 1/4 2/6 <1/1

TABLE XXI

Phenyl benzenesulphonamides R SO₂NH R'

Number	R	R'	In vitro index
2480	amino H methyl H methyl methyl	H	< 1/4
6419		H	3/6
6418		H	3/7
6420		methyl	< 1/7
6417		methoxy	1/7
6416		chloro	4/8

Turning to the results of therapeutic tests, 10 new compounds were selected on the basis of high in vitro activity 4 4'-Diaminodiphenylsulphone and No 4879 (which corresponds to promizole) were also included Before carrying out these tests, six of the compounds whose constitutions lent themselves to the method of analysis were given orally to mice at maximal doses and the blood concentrations determined (Table XXII)

TABLE XXII

Blood concentrations attained after oral administration of maximum tolerated doses to mice

Number	See Table	Dose mg /20 g mouse			ons (mg / ter dosing 4 hr
371 4286 4879 5106 4197 4409	XIV XIV XVI XVIII XVIII XVIII	3 5 10 5 8 9	3 2 12 2 10 0 9 1 2 0 6 9	3 1 4 5 9 1 10 0 1 5 3 6	28 09 69 75 15

We could not be sure that some of the remaining compounds were absorbed to any appreciable extent because no method of analysis was available for them and mice tolerated, without gross toxic effects, relatively large doses. Certain others were definitely absorbed because small doses were toxic to mice, but we have no knowledge of the blood levels corresponding to these doses. The results of therapeutic tests are listed in Table XXIII. As would be expected, activity was found with 4 4'-diaminodiphenylsulphone (No 371) and with 2 4'-diamino-5-thiazylphenylsulphone (No 4879). No activity was observed with any of the others

TABLE XXIII

THERAPEUTIC TESTS IN MICE 24 mice in each group, infected intravenously and treated orally

Number	See Table	Dose mg /20 g	Increased mean sur- vival time	Increase re- quired for significance
3938 3983	X XVII	4 0 3 0	$-18 \\ -07$	1 9 1 9
2985	XX	1 0 2 5 5 0	$ \begin{bmatrix} -0.7 \\ +0.3 \\ -0.6 \end{bmatrix} $	1 15
371	XIV	1 0 2 0 3 0 4 0	+11 +22 +19 +25	1 8 1 7
4286	XIV	50 _	0	2 0
5405	XIV	0 1 0 25	$ \begin{array}{c} +0.3 \\ -0.3 \end{array} $	16
4879	XVI	2 5 5 0 5 0	+08 +22 +17	1 8 1 4
5106	XVI	1 0 2 5	+01+02}	18
5445 4197 3901 4409	XVIII XIX XIX XVIII	10 0 8 0 10 0 8 0	+06 -23 -05 +09	1 8 1 5 1 5 1 5
6416	XXI	1 0 2 5 5 0	$ \begin{array}{c} +0.1 \\ -0.3 \\ -0.8 \end{array} $	_ 1 15

SUMMARY

- 1 The testing of a large number of sulphones and related sulphonates and sulphonamides against Mycobacterium tuberculosis in vitro is recorded
- 2 Therapeutic tests in mice have shown that high in vitro activity does not necessarily lead to activity in vivo

Chemical papers referring to the methods of pre paration of the compounds mentioned here (where these are not already well known) have appeared (Burton and Hoggarth, 1945, Hoggarth, 1947) Some of the compounds listed were originally submitted by Dr Burton, of the University of Leeds, for general antibacterial examination Our thanks are due to him and also to Miss M Scott of these laboratories for help with the preparation of some of the larger samples required for the therapeutic tests statistical assessment of the therapeutic experiments was carried out by Dr O L Davies, of our Statistical Department.

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STUDIES IN THE CHEMOTHERAPY OF TUBERCULOSIS. PART II SULPHONAMIDES

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Rich and Follis (1938) showed that sulphanilamide had a retarding effect upon tuberculosis in guinea-pigs and later (1939) they were also able to demonstrate a slightly favourable response against "bovine" tuberculosis in rabbits Sulphapyridine had no significant effect in the latter experiments, though other workers—for example, Feldman and Hinshaw (1940)—have shown that this drug has some slight action on tuberculosis in guinea-pigs A more favourable effect in guineapigs was obtained with sulphadiazine (though not with sulphapyridine or sulphathiazole) by Smith, Emmart, and Westfall (1942) In all experiments the observed therapeutic benefit has been slight and could be demonstrated only when the drugs were given at maximum tolerated doses Examination of a number of the commoner sulphonamide drugs in mice by the method previously described by one of us (Martin, 1946) is in agreement with We obtained a significant these earlier results increase of survival time with the higher doses of sulphadiazine (No 2052) and with the highest dose of No 3536 (which corresponds to sulphamerazine) but not with the other sulphonamide drugs used (Table II)

Though none of these results is of practical importance, we were led to return to this group of compounds by the remarkable pharmacological properties of 2-sulphanilamido-4 6-dimethoxypyrimidine (No 3445) The preparation of this compound and some homologues has been described by Rose and Tuey (1946), it was developed in these laboratories in the course of a search for improved sulphonamides for the treatment of nontuberculous bacterial infections. It has been shown to be well absorbed, extremely persistent in the body and very efficient in the treatment of streptococcal infections in mice (Gage, Martin, Rose, Spinks, and Tuey, 1947) In vitro the drug No 3445 had only a very feeble activity against Mycobacterium tuberculosis, as indicated by its in vitro index < 1/6 (for description of our method of measuring in vitro act vity, see Hoggarth and Martin, 1948), and failed to show any activity in mice. Our previous experience with sulphones led us to believe that the in vitro activity of drug. No 3445 might be raised by suitable modification.

TABLE I

(a) NH. SO₂NH $\stackrel{N}{\underset{N=R}{\longrightarrow}}$

Number	R	In vitro index
3445	methoxy	<1/6
3706	ethoxy	<1/3
4583	n-propoxy	5/7
4584	isopropoxy	4/8
5105	<i>n</i> -butoxy	5/7
5239	<i>iso</i> butoxy	6/8
5033	l-methylpropoxy	4/7
5066	methylthio	< 1/7

Num- ber	Position of amino group	x	R'	R"	In vitro index
3052	p	SO ₂	methyl	methoxy	<1/1
5162	p	S	methoxy	methoxy	<1/4
4594	m	SO ₂	methoxy	methoxy	2/6

of its structure Provided that the favourable pharmacological properties were retained, it was reasonable to expect activity in 1110

Examination of the *in vitro* indices for a series of 2-sulphanilamido-4 6-dialkoxypyrimidines (Table I) shows that, as expected, the *in vitro*

activity increases with increasing size of the alkyl groups, at least as far as 4 carbon atoms. Prior to examination of these compounds in vivo, the blood levels attained in mice dosed orally with these new compounds were examined by our colleague Dr. Spinks (Spinks, 1947), who found that whilst the property of persisting in the blood stream was retained, the maximum concentrations which could be attained fell off with increasing molecular weight. With the butoxy compounds, the maximum blood level was so small that it seemed unnecessary to carry the synthetical work any further. The in vitro activity of 2-sulphanilamido-4 6-dimeth-

TABLE II
NH. SO.NHR

Num- ber	R	Dose (mg per 20 g mouse)	Increased mean survival time	Increase required for signi- ficance
2052	2-pyrimidyl	10 0 8 0 5 0 2 0	+39 +29 +13 0	1 9 1 7 1 7 1 7
3536	2-(4-methyl- pyrimidyl)	8 0 5 0 2 0	+17 +05 +12	1 7 1 7 1 7
1968	2-(4 6-dimethyl- pyrimidyl)	10 0 2 5	-1 0 -1 1	1 8 1 8
2347 6131	2-thiazyl -CS NH,	10 0 10 0	-111 + 05	1 5 1 1

oxypyrimidine (No 3445) was not increased by replacing the methoxy groups by methylthio groups (No 5066), by replacing one methoxy group by methyl (No 3052), or by replacing the sulphonamide linkage (-SO₂-NH-) by sulphenamide (-S-NH-) (No 5162), though by changing the position of the amino group a slight increase was noted (No 4594) As this last change led to loss of power to persist in the blood (Spinks, 1947), it was not considered that any of these variations applied to compounds with larger alkoxy groups would result in increased activity in vivo

The tests summarized in the following tables were carried out as described in the preceding paper by Hoggarth and Martin (1948) Drugs were given orally, usually twice daily for five days and once on Saturdays, but the remarkable persistence of the 2-sulphanilamido-4 6-dialkoxy-pyrimidines in the blood made it unnecessary to

dose oftener than once daily with some of these compounds Infection was carried out intravenously, using 1 mg of moist growth of "human" tubercle bacillus from a culture on Lowenstein's medium suspended in water

DISCUSSION OF RESULTS

The therapeutic results show (Table III) that in vivo activity against Mycobacterium tuberculosis can be attained by compounds of the 2-sulphanilamido-4 6-dialkoxypyrimidine class Activity in this group seems to be limited by the same con-

TABLE III

NH. OR SO_2NH N = OR

Num- ber	R	Dose (mg per 20 g mouse)	Increased mean survival time	Increase required for signi ficance
3445	methyl	5 0*	+11	14
4583	n-propyl	5 0* 2 5* 2.5	$^{+1}_{-05}$ $^{+20}$	1 4 1 5 1 4
4584	<i>iso</i> propyl	- 7 5* 5 0* 4 0 2 5*	+1 1 +6 4 +4 0 -1 5	1 4 1 4 2 2 1 5
5105 5033 5239	n-butyl l-methylpropyl isobutyl	5 0 5 0 3 0	+01 +12 +10	1 3 1 4 1 3

*Dosed once daily only

siderations as were found to be operative with the sulphones (Hoggarth and Martin, 1948)—namely, that substituents sufficiently large to confer high activity in vitro result in such poor absorption that activity in vivo cannot be expected. This is certainly the explanation, at least in part, of the activity of the n-propoxy and isopropoxy compounds (Nos 4583 and 4584) on the one hand, and the absence of activity in all the butoxy compounds (Nos 5105, 5239, and 5033) on the other, although all show about the same high activity in vitro (Table I)

Our interest in 2-sulphanilamido-4 6-di 150propoxypyrimidine (No 4584), which produced a very striking increase in mean survival time at the optimal dose, was lessened by the observation that this beneficial effect is almost lost when dosing (which normally begins just before infection) was delayed by so little as 24 hours. With other un related compounds (to be reported) we have shown a definite prolongation of life of treated animals even when dosing was delayed for as long as 7 days The anti-tuberculous activity of No 4584 is therefore of no practical significance

SUMMARY

- 1 The activity of a series of 2-sulphanilamido-4 6-dialkoxyprimidines and some closely related compounds against Mycobacterium tuberculosis in vitro has been studied In the former group activity in vitro increases with increasing size of the alkoxy groups
- 2 The therapeutic action of certain of these compounds in mice infected with Mycobacterium tuberculosis was examined The d_{1-n} -propoxy and di-isopropoxy compounds produced a significant increase in the mean survival time of groups

of mice treated with them when the drug was given both before and after the mice were infected When drug treatment was delayed for 24 hours, no therapeutic effect was demonstrated

3 The higher members of the series were very poorly absorbed and failed to show any therapeutic action

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STUDIES IN THE CHEMOTHERAPY OF TUBERCULOSIS PART III ANTIMALARIAL COMPOUNDS

BY

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When we first established our method for examining the antituberculous activity of compounds in mice (Martin, 1946), work in these laboratories which subsequently led to the development of the drug paludrine made available to us a number of new types of compounds showing marked antimalarial activity We examined these by the new procedure, with the result that promising activity was obtained with certain compounds The principal stages in the chemical investigations which led ultimately to the discovery of antimalarial activity in N¹ - p - chlorophenyl-N⁵ - isopropylbiguanide (" paludrine ") have been described by Curd and Rose (1946), and are indicated in Table I below, which also shows the compounds in which antituberculous activity has been found

RESULTS

Therapeutic tests against Mycobacterium tuber culosis in mice have been carried out on each of the main classes of compounds and the results are listed below (Tables II, III, IV) We also thought it proper to examine other well-known antimalarial drugs, no activity was found with quinine or mepacrine at doses of 0.5, 1.0, or 2.0 mg per 20 g mouse, nor with pamaquin at doses of 0.05, 0.1, or 0.2 mg per 20 g mouse. The test method was that previously described (Martin, 1946) Briefly, this consisted in the infection of mice by the intravenous route and their treatment by drugs administered twice daily by syringe and catheter at doses ranging downwards from the maximum tolerated. The results are presented in the form used in

TABLE I

Type of compound		Antituberculous	Representative compound
2-arylamino-4-dialkylaminoalkylamino-6-methylpyrimidines	active	active	CI NH N CH ₃ NH(CH ₂) ₂ NEt ₂ No 2666 (2HCl)
2-arylguanidino-4-dialkylaminoalkyl- amino-6-methylpyrimidines	more active	inactive	CINH C NH NH(CH ₂) ₂ NEI NO 3349 (2HCI)
N¹-aryl-N⁵-alkylbiguanides	very active	ınactıve	CINH C NH C NHPr ⁶ NH NH No 4888 (CH ₃ CO ₂ H) ("paludrine")

TABLE II

Therapeutic tests on 2-arylamino-4-dialkylaminoalkylamino-6-methylpyrimidines given orally to mice infected with Mycobacterium tuberculosis Doses given twice daily, as dihydrochlorides

Ar NH
$$\langle N = NHR \rangle$$

No	Ar	R	Dose (mg per 20 g mouse)	Increased mean survival time (days)	Increase required for significance (days)
2666	p-chlorophenyl	β-diethylaminoethyl	1 0	+23	16
3711	p-chlorophenyl	γ-dimethylaminopropyl	0 5 1 0 1 5 2 0	0 +2 2 +2 0 +1 7	1 7
3299	p-chlorophenyl	γ-diethylaminopropyl	0 5 1 0	+2 6 +3 2	1 9
			1 0 1 5	+3 4 +2 2	1 7
3300	p-chlorophenyl	δ-diethylamino- α-methylbutyl	1 0	+55	1 6
	•	a-methylbutyi	1 5 2 0	+4 2 +4 2	1 7
3502	6-bromo-2- naphthyl	β-diethylaminoethyl	1 0 1 5 2 0	+5 5 +4 0 +2 4	1 4
			1 0 2 0 4 0	+3 9 +3 4 +0 4	1 7

TABLE III

Therapeutic tests on 2-arylguanidino-4-dialkylaminoalkylamino-6-methylpyrimidines given orally to mice infected with *Mycobacterium tuberculosis* Doses given twice daily, as dihydrochlorides

No	Ar	R	Dose (mg per 20 g mouse)	Increased mean survival time (days)	Increase required for significance (days)
3349	p-chlorophenyl	β-diethylaminoethyl	1020	-21 -06	1 8
3672	p-chlorophenyl	γ-diethylaminopropyl	0 1 0 5 1 0	-04 0 -09	17
4926	6-bromo-2- naphthy l	β-diethylaminoethyl	0 25 0 5	-07 -12	17

TABLE IV

Therapeutic tests on N¹ aryl-N⁵-alkylbiguanides (and related compounds) given orally to mice infected with Mycobacterium tuberculosis Doses given twice daily, No 4967 as base, No 4095 as sulphate, No 5114 as carbonate, and the rest as acetates

$$\begin{array}{c|c} \text{CI} & \text{NH C NH C N} \\ & \parallel & \parallel \\ & \text{NH NH} \end{array}$$

No	R	R'	Dose (mg per 20 g mouse)	Increased mean survival time (days)	Increase required for significance (days)
4967	ethyl	Н	0 025	-02	1 6
4887	n-propyl	Н	0 1 0 05	-0 2 -0 4	1 4
4888	<i>iso</i> propyl	Н	0 1 0 25	+0 9 -0 7	1 3 2 2
4565	n-butyl	Н	05,	-01	15 -
4430	n-propyl	methyl	0 25 1 0	+0.5 +0.2	1 3
4095	n-butyl	n-butyl	1 0 0 5	+0 6 +0 3	1 8
5114	δ-diethylamino- α-methylbutyl	Н	10	+03 +08	18

preceding papers of this series (e g, Hoggarth and Martin, 1948)

It was of interest to see how far the therapeutic effects of the new drugs shown in Table II were dependent upon the method of examination. The high activities of compounds Nos 3300 and 3502 made them suitable for this purpose. It was possible to demonstrate a therapeutic effect with both of these compounds when certain modified dose schedules, as shown in Table V, were adopted

It will be seen that compound No 3300 exerted a slight but definite therapeutic effect when dosing was begun one week after infection, showing that the drug was having an inhibitory effect even on established tuberculous disease. The absence of therapeutic effect with a single dose of drug given either immediately before, or 24 hours after, infection is important, since it shows that the drug is not merely killing freshly introduced organisms, but is exerting an effect upon them while they multiply in the tissues

As a further modification of the usual procedure, compound No 3300 was given mixed with the food. The drug was mixed with the powdered food, weighed quantities of which were offered to the mice in special containers designed to minimize loss. The consumption of the drug was estimated

TABLE V
Further therapeutic tests on compounds Nos 3300 and 3502

No	Dose mg per 20 g	Schedule of dosing	Increased mean survival time (days)	Increase required for sig nificance (days)
3502	20	(i) twice daily for first 5½ days (ii) as usual (for 19 days)	4 1 4 0	1 6
3300	10	(i) one dose only be- fore infection (ii) one dose only 24	+08	
		hours after in- fection (in) twice daily for first	-01	
		5½ days after in fection (iv) twice daily from 8	+34	1 4
		to 13½ days after infection	+18	
		(v) as usual (for 16 days)	+35	

by weighing the residual food at daily intervals. The figure given in the "dose" column of Table VI (referring to experiments in which the drug was given by this method) is the estimated average daily

intake The drug was given to other mice by syringe and catheter as usual for comparison During week-ends when dosing by syringe was suspended, the drug-diet was replaced by plain food

TABLE VI

Further examination of compound No 3300 Comparison of the effect of oral dosing with the administration of the drug in the diet. Treatment given for the first 16 days

Dose mg per 20 g	Method of adminis- tration	Increased mean survival time (days)	Increase required for signifi- cance (days)
0 5 1 0 2 0 3 0	ın food (per day)	+07 +32 +56 +48	1 6
0 5 1 0 2 0 3 0	by syringe and catheter (twice daily)	+0 8 +1 4 +4 4 +4 8	10

DISCUSSION

Previous investigations of the chemotherapeutic activity of synthetic substances in tuberculosis have been almost all concerned with compounds of the sulphone-sulphonamide types. The only exceptions of which we are aware are p-aminosalicylic acid (Feldman, Karlson, and Hinshaw, 1947) and certain naphthoquinones (Alcalay, 1947). Attention was first drawn to the compounds reported here because of their antimalarial activity, but it is clear that the two types of therapeutic action are not co-extensive. Furthermore, even in the group

of compounds showing both antituberculous and antimalarial activity, the compounds are placed in a different order of activity by the *in vivo* test against *Mycobacterium tuberculosis* in mice and the antimalarial test with *Plasmodium gallinaceum* in chicks (Curd, Davey, and Rose, 1945, Curd and Rose, 1946a) Thus, whereas No 3300 is more active than No 2666 in the antituberculous test, the reverse is the case in the antimalarial test. The influence of chemical structure upon therapeutic activity in compounds related to No 3300 will form the subject of a future communication.

SUMMARY

Antituberculous activity in mice has been demonstrated with a new group of compounds (2-arylamino-4-dialkylaminoalkylamino - 6-methylpyrimidines), some members of which are active as antimalarial drugs. No activity was found with the other antimalarial drugs tested

Most of the compounds mentioned in this report were prepared by the team of chemists working under the direction of Drs F H S Curd and F L Rose to whom we wish to express our thanks

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STUDIES IN THE CHEMOTHERAPY OF TUBERCULOSIS PART IV DIAMINO METHYLPYRIMIDINES AND RELATED COMPOUNDS.

BY

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We have already reported (Hoggarth and Martin, 1948) that members of a certain class of antimalarial compounds (2-arylamino-4-aminoalkylamino-6-methylpyrimidines) show antituberculous activity in mice. When examined by the standardized procedure used throughout this series of investigations (Martin, 1946) the compound showing most promise was 2-p-chloroanilino-4- δ -diethylamino α -methylbutylamino - δ - methylpyrimidine dihydrochloride (No 3300)

We have examined the relationship between antituberculous activity and chemical constitution among a group of compounds related to the drug No 3300 and present here a summary of the main conclusions which have emerged after a study of 110 such compounds. The chemical formula of compound No 3300 may be generalized as shown below, and we have prepared variants changing each portion of the general structure in turn

 $(Ar_i = an aryl residue, R = an aliphatic residue usually containing another basic centre)$

RESULTS

The test method consisted of the infection of mice by the intravenous route, and their treatment by drugs administered orally, twice daily by syringe and catheter at doses ranging downwards from the

maximum tolerated. The results are presented in the form used in preceding papers of this series

1 Variation of aryl residue (Ar) —Retaining the 4- δ -diethylamino- α -methylbutylamino - 6 - methylpyrimidine portion of the compound No 3300, the arylamino residues listed in Table I were substituted for the p-chloroanilino group In compound No 3656 the aryl nucleus was omitted altogether

TABLE I

Therapeutic tests on some 4-δ-diethylamino α methyl butylamino-6-methylpyrimidines containing an aryl amino group (or in one case an amino group) in position 2 Doses given orally twice daily by syringe and catheter

Compounds of the form

	C	H ₃	_	
No	Ar	Dose (mg per 20 g mouse)	Increased mean survival time (days)	Increase required for signifi cance (days)
5753	p-fluorophenyl	1 0	+1-6	1 4
5207	p-bromophenyl	10	+3 9 +4 1	14
5499	p-iodophenyl	0 5 1 0	+2 2 +3 2	} 1,3
5161	m-chlorophenyl	0 5 1 0 2 0	0 +0.2 +0.4	} 16
5164	o-chlorophenyl	1 0 2 0	-03 -03	} 16

TABLE I (continued)

No					
phenyl	No	Ar ،	(mg per 20 g	mean survival time	required for signifi- cance
	5210		1 0	+30	1 6
phenyl 1 0	5414	2 5-dichloro- phenyl	1 0	-08	1 3
phenyl 1 0 +2 0 1 3 5211 p-anisyl 3 0 +2 0 1 6 5214 o-anisyl 3 0 +0 1 1 6 5548 p-tolyl 0 5 +3 2 } 1.5 1 0 +5 6 } 1.5 1 0 +2 6 } 1.5 5560 m-tolyl 2 0 +2 6 1 5 5828* p-sulphonamido-phenyl 10 0 +0 3 1 4 6259 p-dimethylamino-phenyl 0 5 +0 5 1 1 4977 6-bromo-2-naph-thyl 0 25 -0 9 1 1 4977 6-bromo-2-naph-thyl 0 5 +1 2 1 3 3656* amino 1 0 -0 5 1 6	5588			+1 I +3 7	} 17
5214 o-anisyl 3 0 +0 1 1 6 5548 p-tolyl 0 5	5500		10	+20	1 3
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	5211	p-anisyl	3 0	+20	1 6
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	5214	o-anisyl	3 0	+01	1 6
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	5548	p-tolyl		+3 2 +5 6	} 1_5
5828* p-sulphonamido- phenyl 10 0 +0 3 1 4 6259 p-dimethylamino- phenyl 0 5 +0 5				+26 +33	} 16
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	5560	m-tolyl	2 0	+26	1 5
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	5828*	p-sulphonamido- phenyl	10 0	+03	14
thyl 05 +18 \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \	6259		0.5	+05	} 11
	4977		0 25 0 5		} 13
	3656*	amıno		1 -	} 16

^{*}Administered as free base and not as dihydrochloride

II Variation in the aliphatic residue (R)—Retaining the 2-p-chloroanilino-6-methylpyrimidine portion of the molecule of No 3300, the δ -diethylamino- α methylbutylamino group of this drug was replaced by a number of aminoalkylamino residues and also by a number of simple amino and hydrazino groupings. A number of such variations are shown in Table II

in Simultaneous variation of the aryl residue (Ar) and the aliphatic residue (R)—Nine compounds having both these groups different from those in No 3300 were examined. The substituent groups were those which appeared most favourable from the results summarized in Tables I and Il No compound of outstanding activity was found

and the range of activity was within that shown by compounds given in Tables I and II These results are therefore not reported in detail

TABLE II

Therapeutic tests on some 2-p-chloroanilino-6-methylpyrimidines containing an aminoalkylamino or amino or hydrazino residue in position 4 Doses given orally twice daily by syringe and catheter

Compounds of the form

No	R	R'	Dose (mg per 20 g mouse)	In- creased mean survival time (days)	Increase required for signifi- cance (days)
4874*	β-ammoethyl	Н	1020	$-0.5^{\circ} + 1.6$	} 14
3671	γ-butylamıno- propyl	Н	0 5 1 0	+ 0 3 + 1 6	} 16
3557	y-piperidino- propyl	Н	0 1 0 25 0 5	$ \begin{array}{r} -0.6 \\ +0.8 \\ +2.2 \end{array} $	} 17
			05	+27 +42	} 18
5718	γ-diethyl- amino α- methylpropyl	Н	0 1	+21	1 4
6112	γ-piperidino- α methyl- propyl	Н	0 25 0 5	¬ 0 9 ¬ 1 4	} 11
6330	2-N-morpho- lino-methyl cvclohexyl	Н	0 5 1 0 2 0	+18 +36 +29	} 18
5556	γ-diethyl- amino α β- dimethyl- propyl	Н	0 5	- 08 +16	13
6306	δ-diethyl- amino α- methylbutyl	methyl	0 05 0 1	7 0 3	} 18
5007*	methyl	methyl	05	+05 -11	} 17
4456*	hydrazino	H	10	-12	1 4
4453*	3 B'-dimethyl- hydrazino	H	05	0 0 6] 15

^{*}Administered as free base and not as dihydrochloride

Additional substituents in position 5 of the pyrimidine nucleus -Position 5 of the pyrimidine nucleus of compound No 3300 is unsubstituted, and an examination was made of the effect of introducing aliphatic residues such as methyl, ethyl, and isopropyl, halogen atoms such as bromine, and nitro or amino groups in this position. All compounds so substituted, except that in which the new group was methyl, were completely lacking in activity, as were seven other compounds carrying one or other of these substituents in position 5, but derived from other active compounds of Tables I and II The activity of the compound No 5671, 2 - p - chloroanilino-4- δ -diethylamino - α - methylbutylamino-5 6-dimethylpyrimidine, was equal to or better than No 3300

Positional isomerism —Two series of positional isomers are known. Some members of the 4-arylamino - 2 - aminoalkylamino - 6 - methylpyrimidine series exhibit a degree of antimalarial activity at least equal to that found in the parent series (Curd, Davis, Owen, Rose, and Tuey, 1946) whereas the 4-arylamino-6-aminoalkylamino-2methylpyrimidine compounds are all inactive (Basford, Curd, and Rose, 1946) These two isomeric types may be represented by generalized formulae below.

(Ar=an aryl residue, R=an aliphatic residue usually containing another basic centre)

We have examined 24 compounds derived from these two series, which were prepared with those arylamino and aminoalkylamino residues which previous results had shown to be most likely to give active compounds Certain examples had a methyl substituent as an additional "favourable' group in position 5 These results are not given in detail for although activity was found in both series (in the 4 6 2 series, only with a methyl group in position 5) it was in all instances of a low order Neither of the isomers of No 3300 and only one of those of No 5671 showed activity

Replacement of the imino linkage uniting aryl and pyrimidyl nuclei by a sulphur or an oxygen atom —The effect of this change, which has been shown to result in diminished but still detectable antimalarial activity in the 2-arylamino-4-aminoalkylamino-6-methylpyrimidines and their 4 2 6 isomers (Curd, Davis, Hoggarth, and Rose, 1947), has been investigated in all three isomeric series No activity whatever was found in the

seventeen examples of ethers and thioethers examined

DISCUSSION

The aim of the work summarized here was the discovery of a compound of the diaminomethylpyrimidine class with greater antituberculous activity than that possessed by compound No 3300 This aim has not been realized, and it would appear that in compound No 3300 itself and a number of closely related compounds we have reached the maximum activity possible in this particular chemical group It is noteworthy that the essential structural requirements for antitubercu lous activity in vivo are similar to, but not identical with, those necessary for antimalarial activity For antituberculous activity in mice the diaminomethyl pyrimidine nucleus must have one amino group substituted by an aryl residue preferably containing a para substituent (though some meta substituents confer activity), and the other by a basic alkyl residue, the new basic centre being separated from the imino linkage by a chain of carbon atoms Of the extra substituents which have been tried in position 5 of the pyrimidyl ring, all except methyl have an unfavourable effect These requirements correspond to what has been found necessary for activity against Plasmodium gallinaceum in chicks (Curd and Rose, 1946, Curd, Richardson, and Rose, 1946), except that no member of the 4arylamino-6-aminoalkylamino - 2 - methylpyrimi dine series has shown antimalarial activity. A low degree of antimalarial activity is retained in the 4-arylamino-2-aminoalkylamino- 6 -methylpyrimi dines when the imino link uniting the aryl and pyrimidyl nuclei is replaced by oxygen or sulphur, this change in all three isomeric series abolishes antituberculous activity

SUMMARY

More than one hundred diaminomethylpyn midines and related compounds have been examined for antituberculous activity in mice. The relationship between activity and chemical consti tution is discussed

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THE INSTABILITY OF STILBAMIDINE

BY

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The recent simultaneous appearance of two publications, by Fulton and Goodwin (1946), and by Henry (1946), dealing with the photochemical instability of cis- and trans-stilbamidine in solution shows the existence of considerable divergence of opinion regarding the exact nature of the changes which occur Barber, Slack, and Wien (1943) suggested that saturation of the ethylenic linkage of trans-stilbamidine on irradiation was due to addition of water at the double bond with carbinol formation Henry (1946) showed that this suggestion was inadmissible, and his evidence was strongly in favour of dimerization with formation of a derivative of cyclobutane interpretation of the nature of the saturated irradiation product has recently been confirmed by Fulton and Dunitz (1947) by x-ray analysis and will therefore be assumed throughout the present communication

Fulton and Goodwin (1946) were able to show the partial conversion of cis-stilbamidine into trans-stilbamidine on irradiation, and also deduced that cis-stilbamidine is not converted directly into the saturated product On these points they and the present author are in complete agreement. On the other hand they were unable to find any evidence for the reverse trans $\rightarrow cis$ change, whereas the present author found that partial conversion of trans-stilbamidine to cus-stilbamidine occurred over a wide range of concentrations and temperatures, equilibrium between the two forms being ultimately established under all conditions used The value of the ratio dimer/cis-stilbamidine produced in the early stage of irradiation is determined by concentration and temperature, low temperature is favourable to dimer formation which explains Fulton's (1943) finding that greater on irradiation at winter toxicity developed temperatures than at summer temperatures Furthermore, no evidence was found for the "stabilization" of trans-stilbamidine in dilute solution which Fulton and Goodwin (1946) postulated in order to explain some of their results at

low initial concentrations of cis-stilbamidine low concentrations, equilibrium between the cisand trans-forms is rapidly established, and the equilibrium concentration of the trans-form is such that its rate of dimerization is very slow in comparison with its rate of reconversion to the cis-form, since the latter reaction is unimolecular whereas the former is bimolecular, this is doubtless the explanation of the apparent "stabilization" of the trans-stilbamidine Their failure to obtain precipitation of trans-stilbamidine sulphate on irradiation of 0.05 per cent solutions of cisstilbamidine sulphate is in agreement with the present author's finding that, at low concentrations, precipitation of trans-stilbamidine sulphate is not complete even in presence of a large excess of sodium sulphate, and consequently the normal bromometric method of analysis of mixtures of the two isomers fails to give reliable results in dilute solution With the fluorescence-adsorption technique of Henry and Grindley (1942) no difficulty was experienced in demonstrating the complete reversibility of the cis-trans change at low concentrations, and it was in fact this property which was used to identify cis-stilbamidine as one of the irradiation products

Fulton and Goodwin (1946) state that "It is very probable that the formation of the 'saturated' product by irradiating aqueous solutions of transstilbamidine is due to collision between activated trans- molecules The high absorption coefficient of this compound indicates that a large proportion of the molecules present in an irradiated solution may be activated" In the first place, it is probably unusual for the mean life of a photochemically activated molecule to exceed 10^{-r} sec so that there is little opportunity for any significant accumulation of activated molecules in the solution to occur Secondly. if the saturated irradiation product arises as the result of carbinol formation it is difficult to see why collision of two activated molecules should be necessary Even with dimer formation it is not necessary to make this assumption, as the energy of photochemical activation of a single molecule corresponding to the

wavelength of maximum absorption (329 m μ , 88,000 cals per gram -mol) is sufficient to bring about any ordinary chemical reaction which is likely to occur

Hydrolysis of the amidine groups of stilbamidine was early found to be a dark reaction (Henry, 1945), and the nature of the first hydrolysis protrans-4-amido-4'-amidinostilbene hydrochloride, was clearly stated Recent examination of solutions of stilbamidine which had been stored for three years under various conditions shows that the factors upon which the rate of hydrolysis of the amidine groups primarily depends are the pH of the solution and the temperature Exposure to light appears not to be of primary importance, but may influence the final results through conversion of the trans-stilbamidine to other compounds which may show different susceptibility to hydrolysis and produce hydrolysis products too soluble to be precipitated A pH of 5 suppresses hydrolysis almost indefinitely. Unless the solutions used by Fulton and Goodwin (1946) were alkaline no significant degree of hydrolysis would be expected during their periods of insolation, particularly at the prevailing temperatures in Britain, and no crystallization would be expected

even on prolonged exposure as substantial conversion of trans-stilbamidine to other products would have occurred in the early stages Storage in the dark, at 40° C, of a one per cent solution of the hydrochloride at pH 7 will almost certainly produce a good crop of crystals of trans-4amido-4'-amidinostilbene hydrochloride within three months It may be added that experience here indicates that the method which they employed for detecting hydrolysis would be unsatisfactory for estimation of the extent of hydrolysis, as at low temperatures ammonia is difficult to aspirate completely, while elevation of the temperature is, under the necessary alkaline conditions —even with borax—liable to result in hydrolysis of the amidine groups The only satisfactory method which has been found of estimating ammonium ion in presence of the amidine group was the formaldehyde method of Marcali and Rieman (1946), a separate "blank" being determined for each base encountered. The results of the long period storage tests are given in Table I The drift of pH which occurred (through action on the glass, etc) interferes to some extent with the deductions which can be made from the

TABLE I
HYDROLYSIS OF AMIDINE GROUPS IN SOLUTIONS STORED FOR 3 YEARS

Expt	Compound	Conc	Conditions	p] Init	H Final	Hydrol to N ₃ Cpd	_ Remarks
A ₁ A ₂ B C D ₁	Stilbamidine Hydrochloride Stilbamidine Hydrochloride Stilbamidine Hydrochloride Stilbamidine Hydrochloride Stilbamidine Hydrochloride	1 0 1 0 1 0 1 0	5° C, glass dark 5° C, wax, dark 30–40° C, glass, dark 30–40° C, glass dark 30–40° C glass dark	67 67 37 45 67	6 50 5 98 5 45 5 92 6 75	Nil Nil 17 56 355	Some cryst of N ₁ Some cryst of N ₁ No visible N ₃ cryst Small tuft of N ₃ cryst Heavy crop of N ₂
D ₂ E ₁	Stilbamidine Hydrochloride Stilbamidine Hydrochloride (Soln init insolated 1½ hrs.)	1 0 1 0	30-40° C, wax dark 30-40° C, glass, dark	6 7 6 7	5 52 6 45	2 6 28 2	cryst No visible N ₁ cryst Some N ₃ cryst
E2	Stilbamidine Hydrochloride (Soln init insolated 1½ hrs)	10	30-40° C, wax, dark	67	6 05	5 3	No precipitation
F_1	Stilbamidine Hydrochloride	10	30-40° C, glass, diffused	67	5 50	67	No precipitation
F ₂	Stilbamidine Hydrochloride	10	daylight 30-40° C, wax, diffused	67	5 65	6 3	No precipitation
G_1	Stilbamidine Isethionate	15	daylight 30–40° C, glass, dark	_	6 67	26 5	Some deposit of N ₂
G.	Stilbamidine Isethionate	1.5	30-40° C, wax, dark		6 25	12 0	Some deposit of N
H ₁ H•	Pentamidine Hydrochloride Pentamidine Hydrochloride	10	30–40° C, glass, dark 30–40° C, wax, dark	7 2 7 2	5 65 5 15	-48 13	No precipitation No precipitation

Notes —1 The symbols N₄, N₃ and N₄ denote trans-stilbamidine and trans-4-amido-4'-amidinostilbene hydro chlorides and 4 4'-diamidostilbene respectively

² In Expts E₁ and E₂ the total bromine absorption after the initial insolation was 49 per cent of the original bromine absorption

results, but there is little doubt that pH and temperature are of primary importance in determining rate of hydrolysis and that it is not a surface action, as had previously been suggested (Henry, 1943)

Trans-4-amido-4'-am dinostilbene is more toxic than the parent compound Its formation in the body—for which conditions of pH and temperature would be favourable-from stilbamidine adsorbed and retained for long periods may therefore in part account for the delayed toxic effects which have been observed The occurrence of prolonged storage in the body is supported by recent examination of the urine of kala-azar patients some eighteen months after termination of their course of treatment with stilbamidine App'ication of the fluorescence-adsorption technique of Henry and Grindley (1945), using 08 cc of urine, leaves little doubt that stilbamidine, or a closely related derivative, is still being excreted at a low level (0 005-0 03 mg per 100 cc) In another patient, only 25 per cent of the stilbamidine isethionate (4,650 mg) injected intravenously over a period of three months was excreted in the urine during the course of treatment, and 14 days after termination of the course the rate of excretion was steady at 01 mg per 100 cc

Determination of the site or sites of storage of stilbamidine in the body is complicated by the difficulty of extracting the drug from adsorbing tissue by the usual organic solvents. It is possible that the answer may be provided by hydrolysing completely both tissue and adsorbed stilbamidine with conversion of the latter into the corresponding stilbene dicarboxylic acid, which can be detected and estimated by its fluorescence (cf Henry, 1946), though with no great sensitivity The point is important, in view of the recent use by Snapper (1947) of stilbamidine in the treatment of multiple myelomatosis Pentamidine, which has been extensively used prophylact cally against trypanosomiasis by van Hoof (1947) in the Congo, resembles stilbamidine in being strongly adsorbed by filter-paper, and is probably also stored in the body for long periods

It seems highly probable that similar conditions of adsorption, storage, and slow release apply for the dimer after administration as appear to apply for stilbamidine. It has been shown (Henry, 1946) that the dimer is strongly adsorbed by filter-paper, and can readily be estimated by the fluorescence-adsorption technique because irradiation of a dry spot on filter-paper with short-wave (ca 245 m μ) ultraviolet light causes reversal of dimerization

and production of trans-stilbamidine, which can then be estimated fluorimetrically. Two sheep were injected intravenously, one with 50 mg of stilbamidine isethionate and the other with the same quantity of dimer isethionate, and the rate of excretion was followed for about 24 hours. The results of these tests are recorded in Table II,

TABLE II

EXCRETION OF *Irans-*STILBAMIDINE AND DIMER IN THE
URINE AFTER INTRAVENOUS INJECTION OF 50 MG
INTO SHEEP

Time of injections 8 43 ho

	Urine	Excreted in	urine
Time	vol, c c	mg /100 c c	mg
trans Stilban	IIDINE		
10 25	65	2 0	13
12 30	50	5 5	28
14 15	50	5 0	25
15 20	20	3 5	07
16 15	48	13	07
08 50	42	0.7	03
09 50	50	0.8	04
11 55	60	0 9	05
Total excretion	on, mg		9 2
DIMER]		
10 25	85	2 5	2 1
12 30	45	2 8	13
14 15	60	0.9	05
15 20	53	0.7	04
16 15 .	46	0.6	03
08 25	55	0 45	0 25
11 05	119	0 3	0 35
11 55	55	0 2	0 1
00	į.		

and show that retention of the dimer in the body is closely similar to that of trans-stilbamidine. The estimation of the dimer was carried out by spotting-out the urine (neutralized to litmus with hydrochloric acid) on filter paper, washing radially, exposing the dry spots to short-wave ultraviolet radiation, and comparing the spots so produced with a series of standard spots which had been prepared in sheeps' urine and treated and developed in the same way

The results of the x-ray examination by Fulton and Dunitz- (1947) of the hydrocarbon obtained by complete hydrolysis of the saturated irradiation product and subsequent decarboxylation are of great interest. In the first place—and on the assumption that they first proved that carbinol formation was inadmissible (otherwise cyclobutane formation could have occurred during decarboxylation)—their results conclusively confirm the dimerization theory of the nature of this product, which had previously been deduced from kinetic

and other considerations Secondly, the apparent rectangular, instead of square, shape of the cyclobutane nucleus of the molecule-confirmation of which will be awaited with much interest—has a very important bearing on the photochemical or thermal decomposition of the dimer siderations of bond-force constants and bond lengths (cf Linnett, 1947) decomposition of the dimer would be expected to occur across the longer sides of the rectangle According to the dimensions of the rectangle given by Fulton and Dunitz (1947) (there is an obvious printer's error in the published data) decomposition across the longer sides of the rectangle would involve some 9,000 cal /gram molecule less than decomposition across the shorter sides, and would result in formation of trans-stilbamidine and not cis-stilbamidine There is already some evidence (Henry, 1946) that photochemical decomposition of the dimer produces trans-stilbamidine only, and not cis-stilbamidine or a mixture of the two isomers, actual proof of this point is rendered difficult by the ready interconvertibility of the cis- and trans- forms on irradiation, and by the convertibility of cisstilbamidine into the trans-isomer at high temperatures (Henry, 1945) The rectangular shape of the cyclobutane nucleus is likely to have some bearing upon the question of why cis-stilbamidine shows no tendency to undergo photochemical dimerization whereas trans-stilbamidine does so readily

SUMMARY

1 A number of points arising out of recent publications dealing with the photo-chemical

changes which stilbamidine undergoes on irradiation are discussed

- 2 Hydrolysis of the amidine groups has been shown to be dependent primarily upon the temperature and the pH of the solution. It is a dark reaction
- 3 The dimer has been shown to be retained in the body after intravenous injection in the same way as is stilbamidine itself

The author is greatly indebted to Mr D N Grindley, who has been associated with much of this work. He also expresses his thanks to Dr E S Horgan, for carrying out injections of sheep, to Dr T N Jewitt, for pH measurements—and to the Director, Sudan Medical Service, for permission to publish this paper

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MIRACIL D, ITS TOXICOLOGY, ABSORPTION, AND EXCRETION IN ANIMALS AND HUMAN VOLUNTEERS

BY

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Miracil D is a new compound which has been devised for the treatment of schistosomiasis (bilharziasis). The present paper outlines its behaviour when given to laboratory animals and human volunteers. A subsequent paper will give information about clinical trials of this compound in patients infected with Schistosoma haematobium or S mansoni

HISTORY

The original discovery of the action of miracil against schistosomes was made at the Elberfeld Research Laboratories of the I G Farbenindustrie, where experimental study of infections with S mansoni was begun by Dr W Kikuth in A series of compounds, synthesized by Dr Mauss (known as the miracil series), was tested in 1938 by Kıkuth and Gonnert and found to have considerable activity Further investigation led in 1941 to the conclusion that the greatest activity against schistosomal infections of monkeys was reached in a member of the series designated miracil D (Kikuth, Gonnert, and Mauss, 1946) This compound was investigated pharmacologically by Dr Hecht, who studied it in a limited number of mice, rabbits, and cats, and considered that it might lead to gradual fatty degeneration of the liver, kidney, and heart muscle Owing to the war, clinical trials were impossible and no further progress was made in Germany, apart from tests carried out by Prof Vogel, which showed that S japonicum was not susceptible to these compounds (S haematobium could not be tried) After the military occupation of Germany, this information was discovered by Allied investigators and the compounds were studied further in Britain and America In America, Bueding, Higashi, Peters, and Valk (1947) found that a majority of mice were cured of infections with S mansoni when treated with miracil D in doses of 36 mg per kg, administered

intraperitoneally every 8 hours for 18 doses, these doses killed one-quarter of the mice In Britain the pharmacology of miracil D was studied in small animals by Wood (1947), who did not confirm the insidious toxicity reported by Hecht, and delicate methods for the estimation of the compound in body fluids were worked out by Coxon, Latner, and King (1947) Early in 1947 we began to study its toxicity and behaviour in rabbits, monkeys, and human volunteers, and in July (by the kindness of the Medical Director, Southern Rhodesia) we were able to begin clinical trials Studies on the toxicity have also been carried out by workers at the Wellcome Research Institute under Dr J S K Boyd To all the above workers we are grateful for kindly giving us confidential information of their results as they were obtained

CHEMISTRY

Miracil D is the hydrochloride of 1-methyl-4- β -diethylaminoethylaminothioxanthone

It is a crystalline orange-yellow powder which is soluble up to 1 to 2 per cent in water at room temperature. When present in body fluids it can be estimated by alkalinization and extraction first into ether and then into dilute hydrochloric acid. The yellow colour of the resultant concentrate is estimated at a suitable pH in a Spekker absorptiometer which has been calibrated by means of known concentrations of miracil (Coxon, Latner, and King, 1947). The sensitivity of this technique is sufficient to detect 0.05-0.1 mg per litre.

PHARMACOLOGY

Miracil has an irritant action when applied locally to the tissues, and subcutaneous or intramuscular injection causes considerable inflammation and some necrosis. When it is injected intravenously, its toxicity is much greater than when it is given by mouth and it tends to cause thrombosis of the vein. Consequently, oral administration is to be preferred.

According to Kikuth and Gonnert (1945, 1948), Hecht (1945), and Wood (1947), the maximum single doses tolerated by mice are 300 to 1,000 mg per kg by mouth, 340 to more than 500 mg per kg by subcutaneous injection, and 20 to 30 mg per kg by intravenous injection Rabbits tolerate single doses of 600 to 800 mg per kg by mouth, but only 15 to 20 mg per kg by intravenous in-With repeated oral doses, mice tolerate 125 mg per kg daily for 10 days Wood found that rabbits died after 4 daily doses of 150 mg per kg or 6 daily doses of 50 mg per kg. In our own experiments rabbits tolerated 28 daily oral doses of 50 mg per kg but died after 12 or 14 daily doses of 100 mg per kg One monkey (No 70) weighing 35 kg survived a total dose of 5 45 g per kg by mouth, given as 3 doses of 50 mg per kg in 6 days, 13 doses of 100 mg in 30 days, and 20 doses of 200 mg in 30 days Another monkey (No 105) weighing 46 kg survived a total dose of 3 4 g per kg, given as 17 doses of 200 mg per kg in 26 days A third monkey (No 94) weighing 36 kg died after 48 g per kg given as 12 doses of 400 mg in 18 days. A fourth (No 72) weighing 3 2 kg died after 5 g per kg given as 13 doses of 200 mg in 30 days and 6 doses of 400 mg in 9 days Apparently the maximum tolerated dose for monkeys is about 200 mg per kg four times a week The minimum curative dose for mice infected with S mansoni is 120 mg per kg on 6 successive days, while monkeys have been cured by two oral doses of 10 mg per kg (Kikuth and Gönnert)

Wood reports that intravenous injections into rabbits of more than 20 mg/kg quickly cause convulsions, similar to those of picrotoxin or strychnine, often there is head retraction and extension of the limbs. In anaesthetized rabbits or cats small doses cause no particular effect on the cardiovascular system, larger doses cause depression of the heart and dilatation of the peripheral vessels. Other investigations on isolated organs have shown no significant pharmacological actions, except a mild spasmolytic action on intestinal muscle.

ABSORPTION, DISTRIBUTION, AND EXCRETION

For this work we have estimated miracil in the body fluids by the method of Coxon, Latner, and King (1947) In one rabbit which had received 0.8 g per kg by mouth, the blood concentration of miracil was 1.6 mg per litre at 24 hours, 1.1 mg at 48 hours, and 0 at 144 hours. Fig 1 shows the curve of the blood concentration after a single dose of 0.4 g per kg by stomach tube to a monkey (No 94) Absorption was rapid and the concentration in the blood was sustained for at least 21 hours

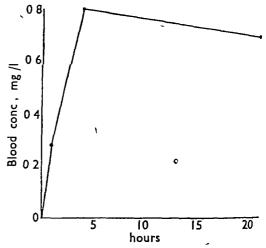


Fig 1—Blood concentrations of miracil in a monkey (No 94) after a single oral dose of 0 4 g per kg

In 3 monkeys which were receiving oral doses of 01 to 04 g per kg, daily or every other day, the blood concentration 24 hours after the previous dose was 0 65, 0 80, and 1 60 mg per litre respec tively, the concentrations not being in proportion to the dosage administered In a monkey (No 70) which had been receiving 02 g per kg (20 doses in 30 days) the blood was examined 3 days after the last dose, and it contained only a trace of miracil (0-0 1 mg per litre) Since 10 mg per kg, repeated once, is stated by Kikuth and Gonnert to be the minimum curative dose for monkeys, this dose was given to a monkey weighing 23 kg, at 21 hours the blood concentration was just detect able (about 0 1 mg per litre), and at 6 hours miracil could not be detected Another monkey weighing 27 kg was given 20 mg per kg, at 2½ hours the blood concentration was 0 45 mg per litre. In a third monkey, given 20 mg per kg, no miracil could be detected in a 25 cc blood sample (1e probably less than 0.1 mg per litre) These con centrations should be compared with those ob tained in the blood of human subjects (below)

Samples of urine collected from some of these monkeys contained 33 to 64 mg per litre. The monkeys in these experiments passed about 100–150 c c of urine per day. When the administration of miracil to two of the above monkeys was discontinued the excretion of miracil in the urine diminished rapidly and ceased after 3 days or 4 days respectively. The faeces of the monkeys which had received these repeated high doses contained 3 8 to 10 mg miracil per g moist weight

The distribution of miracil in the different organs was studied in two monkeys which died from prolonged overdosage. Monkey No 94 had received 12 doses of 400 mg per kg during 18 days, and monkey No 72 had received 13 doses of 200 mg per kg in 30 days, and 6 doses of 400 mg per kg

TABLE I

THE CONCENTRATION OF MIRACIL AND ITS DEGRADATION PRODUCT IN THE ORGANS OF TWO MONKEYS

Concentrations in mg per kg

Ratio = Concentration of degradation product
Concentration of miracil

0		Mir	acıl				datio duct	Ratio				
Organ	No	94	No	72	No	94	No	72	No	94	No	72
Brain Muscle Liver Heart Kidney Lung	30 25 21			3 4	8	8	140 9	2 2	17 0 (3 2 0 2 0 (0 (29 09	12 0 0	41 61 23 08

in 9 days The concentrations of miracil extracted are shown in Table I In addition, some of the organs yielded considerable quantities of a vellow pigment which was extracted by ether but did not pass from ether into hydrochloric acid, presumably this was a degradation product of miracil in which the basic character of the molecule had been The high concentration of masked or destroyed miracil in the kidney is presumably due to excretion of the compound in the urine The concentrations in the lung and heart muscle are higher than might have been expected, while that in the liver is lower. The high concentration of presumed degradation product in the liver is easily understood, since the catabolism of miracil probably takes place in this organ, its high concentration in the brain is less easy to explain

Miracil was given to six volunteers, one receiving it on two occasions. The miracil was taken as solution, preferably after a meal, and a piece of bread was eaten to provide extra material in the stomach and protect the gastric mucous membrane The blood concentration curves during the first six hours are shown in Fig 2 and the dose schedules and blood concentration of different subsequent times are given in Table II Fig 2 shows that absorption of miracil is rapid, a relatively high blood concentration being reached in 21 hours, during the next 4 hours, removal of the drug from the blood (by degradation, excretion, etc.) may or may not be greater than the continued absorption from the intestine There are considerable differences between different individuals in the height and persistence of the blood concentration after a

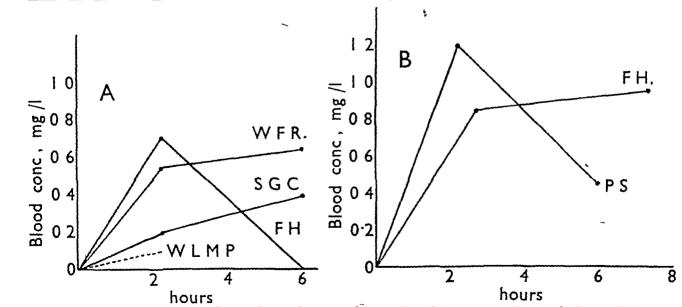


Fig 2—Blood concentrations of miracil in volunteers after single oral doses A—100 mg (volunteer W L M P, shown by dotted line, received 50 mg) B—200 mg

TABLE II

THB BLOOD CONCENTRATION OF MIRACIL ON VARIOUS DOSE-SCHEDULES

The other figures give the blood concentrations of miracil in mg per littre The figures in bold type give the individual doses in g

D.		Symptoms			None	Slight abdominal	uneasiness	Nausea, vomiting, and	latigue	on N		Marked	nausea	fatigue	Slight		Marked	nausea,	etc ,
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3. T	7th	7																	-
	6th	-											0 30	<	-				
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The crosses mark the approximate times when symptoms were felt	4th day	hours	24		İ					06 0			1 20		06 0	×			Υ
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OXIM			<u> </u>		Ť	···		···	01		-	0.7		0 05			0.1		
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보 다			9	<u> </u> 	1	X	1_	×	0.1		4	0 2		0		-	0		
es ni	2nd day	hours	4	1	+		+-	×	10					0 05		 	 		
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-	- <u> </u>		0 0	<u> </u>	<u> </u>	×	<u> </u>	×	10	<u></u>		<u> </u>		a		1			
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_	1st day	hours	•	0	1	0 95	<u> </u>		10	90	- 1 9	07/	0 45	5 0 05			5/	0 20 0 40	-
	Is	٦	77	0.70	1	0 85			-	0 55			1 20	0 0 0 0 05	0 10	ļ.		7 0 —	_
			0	0.1	5	4	03		0.1		,	70							_
	Subject	dosage		FH 01g once	HH	02g once	RH	ອວພວ ສີ ເວ	WFR	daily	0 0	02g twice	daily	WLMP 005 g 3	times daily	SGC	0183	times daily	

given dose Absorption from the intestines is almost complete, and little appears in the faeces In one volunteer (FH, after a single dose of 100 mg) the faeces during 48 hours contained less than 3 mg, in another (WFR, taking 100 mg twice daily) the faeces during the first 30 hours contained about 2 mg, in a third (PS, taking 200 mg twice daily) the faeces collected during the first 48 hours contained 33 mg, some of this may have been excreted into the bowel In volunteers taking repeated doses the blood concentrations on the second day were usually much higher than the corresponding ones on the first day, but after that there was no constant tendency for the blood concentration to rise higher, or for accumulation to occur (Table II) When the drug is stopped, it disappears from the blood in two or three days The amount of miracil excreted in the urine often amounts to about 7 per cent of the dose ingested Since less than 10 per cent of the dose can be recovered from the urine and faeces, it appears that most of the drug absorbed is broken down in the body so that it is not recognized by the test The concentration in the urine may be employed as high as 30 mg per litre, but usually it is much When administration of repeated doses of the drug is stopped, miracil ceases to appear in the urine after 3 days

To study the distribution of miracil between the different elements in the blood two successive samples were taken from volunteer PS after the 5th and 7th doses respectively. In the first experiment, the concentration of miracil in the different components was —

Plasma sample (approximately 44 per cent of total volume), 1.2 mg per litre

Red blood corpuscle sample (approximately 44 per cent of total volume), 0 7 mg per litre

Intervening sample, including buffy coat WBC (approximately 12 per cent), 30 mg per litre

In the second experiment, the concentrations were —

Whole blood, 12 mg per litre

Plasma (63 per cent of the volume), 15 mg per litre

Red blood corpuscles (36 per cent of the volume), 0.5 mg per litre

Buffy coat layer (1 per cent of the volume), 70 mg per litre

The figure for the buffy coat (leucocyte) layer in the second experiment was obtained by calculation from the preceding figures and is not reliable These provisional results indicate that the concentration in the plasma is approximately double that in the red blood corpuscles and that the concentration in the leucocytes (or platelets) is probably much higher, however, the leucocytes contain only a small proportion of the total amount present in the whole blood, since their volume is small.

TOXIC EFFECTS OF MIRACIL

In rabbits, repeated large oral doses of miracil. eg, 04 g per kg daily, cause death after eight or more days In the rabbits which eventually died there was marked loss of weight, haemo-concentration, and occasionally albuminuria There was no anaemia or leucopenia. At autopsy the body fat and other tissues were often stained yellow (presumably with miracil), the kidneys sometimes appeared unhealthy, and the liver might be yellow and might contain white patches Histological investigation revealed, in one rabbit, marked degeneration (sometimes going on to necrosis) of the cells of the renal tubules and areas of necrosis in the liver, in most of the rabbits, however, the degenerative changes in the kidneys and liver were quite mild, and in some they were inapparent The other organs, including the heart, showed no pathological changes

Four monkeys were given repeated oral doses Vomiting often occurred after the early doses, but it ceased later when the same amount of miracil as before was given with a smaller volume of fluid (suspension in gum acacia) The monkeys showed intermittent diarrhoea, but the significance of this is doubtful as many other monkeys in the same room also showed it Two of the monkeys gained 3-6 per cent in weight, two lost about 10 per cent while under treatment Two of the monkeys died had shown no particular clinical symptoms except that on the day preceding death there had been marked lassitude and anorexia The other one developed epileptiform fits, recurring at intervals, and, as death seemed imminent, it was killed There was no anaemia, leucopenia, or albuminuria in these monkeys At autopsy, the abdominal organs were stained yellow, especially the liver Histological examination revealed slight degenerative changes in the renal tubules of one monkey, in the other monkey the kidney showed nothing The other organs, including the liver abnormal and heart, showed no significant pathological

In man, overdosage of miracil seems to produce a different picture It was given to six volunteers as described above, the dosage being raised until symptoms appeared, their weights ranged from 64 to 80 kg A single dose of 02 g (FH) caused some uneasiness of the stomach during the second twenty-four hours after it A single dose of 0 3 g was taken by R H at 10 am, and during the first day he felt tired, he suffered from insomnia during the second part of the night Next morning he felt nauseated and at midday he vomited Later he had slight diarrhoea and felt tired and irritable, but he slept well that night. The third day he was all right again (Dr J S K Boyd has kindly given information about two other volunteers on single One took 02 g with no ill-effects doses other took 04 g about 2 pm That evening there was slight gastric discomfort which continued the next day Sleep was disturbed Forty-eight hours after taking the dose severe nausea, diarrhoea, and retching set in and persisted until the following after which gradual improvement occurred) Volunteer SGC received 01 g three times daily for three days On the second night he had insomnia On the evening of the third day he felt very tired with some aching of the legs and dizziness The sclerotics were yellow During the third night he awoke with headache, pains in the limbs, nausea, and great restlessness, he felt very During the fourth day he had headache, extreme lassitude, nausea, and stayed in bed all day, but his appetite was fair These symptoms passed off gradually and by the sixth day (third day from the last dose) he was normal again Volunteer PS took 02 g twice daily for 7 doses He slept badly on the second night. On the fourth day he felt increasing nausea and fatigue and that night slept badly His skin and sclerotics were yellow On the fifth day the general nausea and malaise continued, on the sixth day his condition improved and by the eighth day he was normal again

Summary of the symptoms in volunteers—After single doses there was no immediate effect on the stomach or intestines and the symptoms usually did not appear until after a latent period of 18-24 hours As can be seen from Table II, the occurrence of symptoms is not related to the concentration of miracil in the blood The principal symptom was nausea, often profound, but vomiting was rare There was also tiredness, prostration, and headache Insomnia occurred in most volunteers, suggesting cerebral excitation skin and sclerotics were often yellow (apparently direct staining) and the urine was bright yellow Two of the volunteers noted pains in the back or Diarrhoea was rare, in fact most of the volunteers tended to be constipated There was no evidence of leucopenia, albuminuria, or jaundice As it has later been found that patients can tolerate 0 6 g or more daily without severe ill-effects, it is possible that some of the above symptoms in volunteers may have had a psychological element

DISCUSSION

The above work was undertaken in order to obtain a provisional picture of the behaviour of miracil in monkeys and man so that the thera peutic action of the compound in patients infected with schistosomes could be examined safely and easily, more detailed investigation of its pharmacology would depend on the outcome of these clinical trials. The following account of the behaviour of miracil must be regarded as only tentative

Miracil is rapidly and completely absorbed from the alimentary canal, only a small proportion appears in the faeces, and this may be due to excretion by the bile or intestine. Excretion in the urine accounts for up to 7 per cent (approx) of the dose. Degradation of the compound in the body is fairly rapid, and on repeated doses there is no obvious accumulation of the compound in the blood after the first day. When administration of the drug is stopped, the body gets rid of the remaining drug in about three days. In the blood, the concentration in the plasma is approximately twice that in the red blood corpuscles, the concentration in the leucocytes is probably much higher

The toxic effects, produced by deliberate over dosage in animals, involve principally the kidneys and liver, they occur only after large and repeated doses, and even then they are often slight. In our experiments we have seen no evidence of the toxic effects on the heart which Hecht reported, and we believe that he was mistaken. In man the symptoms are apparently not due to a direct action of miracil on the stomach or intestines, they seem to be due rather to some degradation product of miracil acting perhaps on certain parts of the nervous system so as to produce nausea and the other disturbances described. The symptoms are unpleasant rather than dangerous

SUMMARY

- 1 Miracil is a new compound, synthesized by Mauss and stated by Kikuth and Gonnert to be highly effective in the treatment of mice and monkeys experimentally infected with Schistosoma mansoni Chemically it is the hydrochloride of 1 methyl 4-β-diethylaminoethylaminothioxanthone It is administered by mouth
- 2 Rabbits tolerate repeated daily doses of 50 mg per kg and monkeys tolerate 200 mg per kg four times a week

- 3 Its behaviour after oral administration has been examined in six volunteers. Apparently it is rapidly absorbed from the alimentary canal, and, after single doses of 0 2 g, the concentration in the blood rises to about 10 mg per litre at 2½ hours Over 90 per cent of the drug is degraded in the body and only about 7 per cent is excreted in the There is little tendency for the drug to accumulate in the body
- The concentration in the plasma is about twice that in the red blood corpuscles, the concentration in the leucocytes is probably much higher
- 5 In animals, deliberate prolonged overdosage may produce degenerative changes in the liver and the renal tubules, but these are usually less than would be expected
- In volunteers the maximum tolerated dose for repeated administration was about 02 g per day Overdosage produced nausea and general prostration, insomnia and yellow discoloration of

the skin and sclerotics also occurred These symptoms came on after a latent period of about one

Grateful acknowledgment is due to the previous workers on miracil, mentioned in the paper, for confidential information about their results, and especially to Dr R V Coxon, Dr A L Latner, and Prof E J King for the instruction in the technique of estimating miracil, to the volunteers who experienced unpleasant discomfort in order to assist these investigations, and to Mr R Hunt for technical assistance

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COMPARISONS OF VARIOUS HISTAMINE ANTAGONISTS

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The experiments described below were undertaken in order to obtain quantitative comparisons of the actions of various histamine antagonists on various different tissues. Consideration has also been given to methods of carrying out two special types of experiment the estimation of histamine antagonists by biological assay, and their use to aid the identification of histamine in tissue extracts and other fluids (Pellerat and Murat, 1945)

In order to test the specificity of the various antagonisms a number of drugs have been used to cause effects which were subsequently suppressed by the different antagonists. These active drugs included histamine, acetylcholine, potassium, nicotine, and adrenaline In discussing these experiments the need has been felt for a collective term to describe these active drugs, and the word "agonist" has been adopted with some hesitation for this purpose It may perhaps also be convenient in discussing other forms of antagonism to use this word to describe the active drug (acetylcholine, adrenaline, p-amino-benzoic acid, etc) and to use the word antagonist to describe the drug (atropine, ergotoxine, sulphanilamide, etc) which suppresses the action of the agonist.

METHODS

The formulae of the various antagonists used are shown below

Concentrations of histamine are calculated in terms of histamine base and those of the other drugs either as molar concentrations or in terms of the salt used (nicotine tartrate, neoantergan maleate, antistin methane sulphonate and hydrochlorides of the other drugs)

Pieces of guinea-pig's ileum, 2-3 cm long, were aerated in a 2-c c bath containing Tyrode's solution. The bath was connected with two flasks so that it could be filled either with ordinary Tyrode's solution or with a similar solution containing the antagonist.

Pieces of uterus from rabbits, cats, and guinea pigs were suspended in a 40 c c bath filled with Dale's solution Some of these uteri had been stored over night at 4° C.

Isolated hearts from rabbits and cats were per fused with thoroughly oxygenated Locke's solution by Langendorff's method. The drugs were injected through the rubber tubing, close to the aorta. In all these experiments the temperature was 37° C.

Experiments on frog's plexus anaesthesia were carried out at room temperature by Sollmann's method as described by Bülbring and Wajda (1945)

RESULTS

Guinea-pig s ileum

Much previous work on the action of antihistamine compounds on isolated intestine has been devoted to antergan, benadryl and pyribenzamine, which are not included in this study (Halpern, 1942, Loew et al, 1946, Winder et al, 1946, Halpern and Mauric, 1946), Bovet (1944) and his collaborators give data for neoantergan Meyer and Bucher (1946) found that antistin was more active against histamine than against acetyl choline. According to Halpern and Ducrot (1946) 3277 RP is not much more active against histamine than antergan, but Winter (1947) reports that 3277 RP is less active than neoantergan and more active than benadryl

The results of such investigations are likely to depend to some exent on the design of the experiment. The method proposed by Schild (1947) appears to be particularly satisfactory, and has been adopted here. The action of the agonist is

first tested in ordinary Tyrode's solution, and a number of equal submaximal effects obtained at regular intervals A similar solution containing the antagonist is then used to fill the bath and the dose of the agonist is doubled The response may be increased at first, but it gradually diminishes and usually reaches a steady level in about 15 minutes The object of the experiment is to find a concentration of the antagonist such that these final responses after 15 min are equal to the original responses to half the dose in the absence of the The whole experiment is repeated until this concentration is found The negative logarithm of the molar concentration which has this effect is defined as the pA₂. Two tests with different drugs are shown in Fig 1 In the first test the concentration of the antagonist was too low and in the second it was too high

The pA₂ can be determined by plotting the results by the method shown in Fig 2, in which

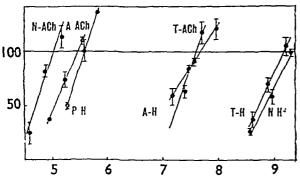


Fig 2—Guinea-pig's intestine Ordinates mean response (4-10 tests) to a double dose as percentage of the response to a single dose without antagonist Abscissae negative log molar concentration of antagonist ACh =acetylcholine H =histamine N =neoantergan T = 3277 RP A =antistin P = nupercaine Vertical lines show ± the estimated standard error

the final response is calculated as a percentage of the initial response and plotted against the logarithm of the concentration of the antagonist The figure corresponding to 100 per cent on this graph is the pA2, and is determined by interpolation, the points can be satisfactorily fitted by straight lines The points plotted in Fig 2 are mostly means obtained from several experiments and the Figure shows standard errors calculated from the individual results, these show that the results are reasonably reliable, but more satisfactory evidence of the error of the method can be obtained by comparing independent estimates of the pA, using different pieces of gut. A few such estimates are shown in the Table, these results confirm the value of the method, and show the high potency of neoantergan and 3277 RP The mean relative molar activities of the various drugs against histamine were nupercaine 1, antistin 125, 3277 RP 4,000, and neoantergan 5,500 Neoantergan is also much the most specific of these drugs. The ratio of its activity against histamine to its activity against acetylcholine was 19,500 The corresponding figure for antistin was 159, and for 3277 RP it was 365

The procedure involves the assumption that after 15 min the response to the agonist would remain unchanged. The fact that this is not certain may account for some of the variations in the results. Duplicate tests on the same piece of gut were sometimes done when the effect of the antagonist was quickly reversible and the inhibitions in such experiments usually agreed within 10–15 per cent. The time for recovery after 3277 RP was longer than after the other drugs

The biological assay of neoantergan —The above procedure would be a slow way of making biological assays of antihistamines These can be carried out by giving a series of equal doses of histamine and observing the depression of the responses due to a brief addition of the antagonist to the bath Fig 3 shows that the effect of 0 002 µg of neoantergan could be distinguished in this way from that of 0 001 µg in the 2-c c bath The effect was not always of this type, since the maximum depression sometimes occurred in the second response after the action of the antagonist In judging the magnitude of the whole effect it is probably best to consider not only the maximum depression, but also the duration of the depression, since this was also increased when larger doses were used possible in this way to make a rough assay of very small amounts of neoantergan, but care is needed, since the inhibition sometimes becomes weaker on successive additions of neoantergan (cf. Ackermann and Maurer, 1944)

The use of neoantergan as a specific test for histamine—When the response of a piece of gut to a tissue extract is abolished by neoantergan this fact may be taken as evidence that the effect of the tissue extract was due to histamine, but the evidence is only convincing if the concentration of neoantergan is very low, since high concentrations abolish the responses to most drugs. A satisfactory method of carrying out tests of this kind is first to find doses of the extract and of histamine which cause equal effects, and then to continue giving these doses alternately and to study the effect, in the series of responses, of a brief addition (1 min) of a small dose of neoantergan to the bath. The dose of neoantergan is chosen so as to pro

TABLE

Values of pA₂ for guinea-pig's ileum—the negative logarithm of the molar concentration of antagonist which halves the sensitivity. Individual values, followed by value from Fig. 2

Antagonist	Histamine		Acetylcholin	Difference	
Neoantergan	9 21, 9 41, 9 49 9 29 9 29	9 32	4 95, 5 04 5 19	5 03	4 29
RP 3277	9 07, 9 09 9 21	9 18	7 67, 7 68	7 62	1 56
Antistin	7 55, 7 66 7 95, 7 95, 7 95	7 67	5 46, 5 57 5 58, 5 55	5 47	2.20
Nupercaine	5 52, 5 7	5 58			
Neoantergan Benadryl Pethidine Atropine	Resu	lts by Schild (1 9 46 8 02 6 13 5 64	1947)	4 86 6 57 5 84 8 61	4 6 1 45 0 29 - 3 03

duce 50-70 per cent inhibition of the subsequent response to histamine The concentration of neoantergan for this effect is usually about 1/10 of the concentration of histamine

Such an experiment is illustrated in Fig 4, which shows that the action of neoantergan on the responses to the extract and to histamine were about equal both in magnitude and in duration Such results provide evidence that the effect of the tissue extract was due to histamine This is further illustrated in Fig 5 In the first experiment shown there the extract was first compared both with histamine and with acetylcholine The addition of neoantergan to the bath for 1 min abolished the response to the extract and to histamine, but did not diminish the response to acetylcholine In the second experiment neoantergan almost abolished the response to histamine but had little effect on the response to nicotine In another experiment the response to nicotine tartrate (1 mg/1) was unaffected by concentrations up to 400 μg/l of neoantergan, but partially inhibited by a concentration of 4,000 μ g /1, while a concentration of $1 \mu g / 1$ was sufficient to produce a marked and prolonged inhibition of equivalent responses to histamine In another experiment the agonists were histamine (10 μ g /1) and KCl (800 mg /1) The effect of histamine was completely abolished by neoantergan in a concentration of 10 μ g /1 The effect of KCl was unaffected even when the concentration of neoantergan was increased to 1 mg/l and was only partly inhibited when the concentration was 5 mg/l The last result is shown in Fig 5C

Isolated uterus

Certain antihistamines themselves cause a contraction of the uterus which complicates experiments on their antagonism to histamine (Halpern, 1942). This antagonism has, how ever, been shown when the direct effect was abolished by leaving the antagonist in the bath for 1-2 hours (Bovet and Walthert, 1944), or by reducing the calcium concentration of the fluid (Halpern and Walthert, 1945), or by using small doses of the antagonist (Dews and Graham, 1946). Thiodiphenylamine compounds, such as 3277 RP, have been found to have no effect by themselves, but to antagonize histamine (Halpern and Ducrot, 1946).

Non-pregnant cat — Neoantergan did not cause contraction of these uteri in concentrations up to 4 mg/l Histamine (15–150 μ g/l) caused contractions which were abolished by equal concentrations of neoantergan Lower concentrations of neoantergan (1/10 that of the histamine) still reduced the response by about 50 per cent Higher concentrations were needed to interrupt an established histamine-response Responses due to acetylcholine were unaffected by neoantergan in concentrations which abolished equivalent responses to histamine

3277 RP (3 mg/l) had no effect by itself, and did not interrupt an established response to histamine If added 1-2 min before histamine in equal concentration, it had little effect on the first subsequent response, but inhibited the second response completely Recovery of the response to histamine was slow

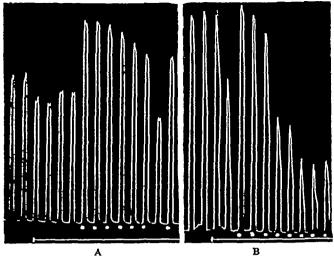


Fig 1—Guinea-pig's intestine Schild's method of testing antagonists Responses to acetylcholine, 12.5 μ g /l Dose doubled at times marked with a dot Bath contained the antagonist at the times marked with a line The effect of (A) neoantergan (2,500 μ g /l) was less than that of (B) 3277 RP (25 μ g /l)

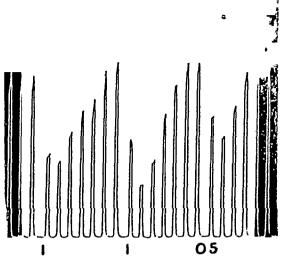


Fig 3 —Guinea-pig's intestine Assay of neoantergan Responses to histamine (10 $\mu g/l$) at intervals of 1 min Inhibitory effects of neoantergan for 1 min Effect of 1 $\mu g/l$ greater than that of 0.5 $\mu g/l$

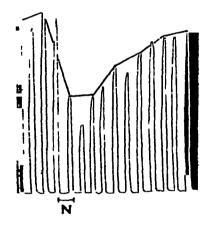


Fig 4—Guinea-pig's intestine Test to identify histamine in an extract Responses at intervals of 1 min. The line joins responses due to 0.2 c.c. of urine extract. Other effects are due to histamine (7.5 μ g/l) except the first (7 μ g/l) N = neoantergan (0.8 μ g/l) for 1 min. This causes roughly equal inhibitions of the effects of the extract and of the histamine given in alternate doses

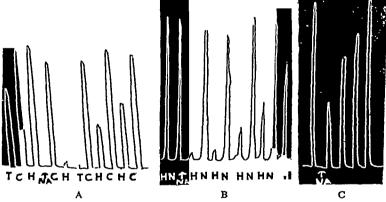


Fig 5—Guinea-pig's intestine NA indicates neoantergan administration A—Neoantergan (0.5 μg /1 for 1 min) inhibited the response to histamine (5 μg /1, H) and 0.2 c c of the extract (T), but not that to acetylcholine (25 μg /1, C) B—Neoantergan (10 μg /1 for 1 min) inhibited the response to histamine (10 μg /1, H) much more than that to nicotine (1 mg /1, N) C—Neoantergan (5 mg /1 for 1 min) inhibited the response to KCl (0.8 mg /ml)

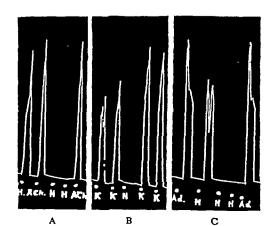


FIG 6—Uteri in 40 ml bath A—Guinea-pig Neoantergan (0.5 μg for 1 min) inhibited the response to histamine (10 μg) but not that to acetylcholine (10 μg) B—Guinea-pig Neoantergan (100 μg) increased the response to KCl (60 mg) C—Rabbit Neoantergan (15 μg) inhibited the response to histamine (25 μg), but not that to adrenaline (25 μg)

The antihistamine activity of benadryl was clearly shown, though less than that of neoantergan On the other hand, benadryl showed pronounced antagonism to acetylcholine

Rabbit—Both pregnant and non-pregnant uteri were used and no difference was seen between them Neither neoantergan nor 3277 RP had any direct effect in concentrations up to 3 mg/l. The response to histamine (17 mg/l) was completely inhibited by neoantergan (0.35 mg/l) added 1 min previously. Recovery occurred after several washings and the inhibition could be repeated. In another experiment, neoantergan (0.7 mg/l) inhibited an established response to histamine (1.7 mg/l).

Antistin was a less powerful histamine-antagonist than neoantergan on this preparation as it was on other preparations

Rabbit uterus differs from the intestine and the other uteri used in that it is stimulated by adrenaline, so that it provides an opportunity for studying adrenaline-antagonisms Fig 6C shows that the response to adrenaline was unaffected by a dose of neoantergan which abolished the response to histamine. Neoantergan $(1,700 \, \mu g / 1)$ had no effect on the response of this tissue to acetylcholine $(70 \, \mu g / 1)$, but 3277 RP in the same concentration completely abolished the response to acetylcholine as well as that to histamine. Benadryl $(350 \, \mu g / 1)$ was also active against acetylcholine

Non-pregnant guinea-pig — Neoantergan had no direct effect in concentrations of 1 mg/l or less, but a concentration of 5 mg/l caused a small contraction. In lower concentrations (16–50 μ g/l for 1 min) it abolished the response to histamine (300 μ g/l) but not that to acetylcholine (cf. Fig 6A). It did not inhibit the response to KCl, but increased it when high concentrations (2.5 mg/l) were used (see Fig 6B). 3,277 RP also antagonized the response to histamine

Isolated heart

Experiments on the heart were in progress when the paper of Dews and Graham (1946) appeared These authors found that neoantergan antagonized the action of histamine on the rabbit's auricle and on the coronary flow in isolated hearts from cats and dogs

Cat s heart —The injection of $0.3-0.5~\mu g$ of hist-amine increased the coronary flow and larger doses $(5-10~\mu g)$ increased the amplitude and rate of the beat. All the antihistamine drugs tested (antergan, neoantergan, benadryl, and 3277 RP) increased the coronary flow and depressed the force and rate of the beat. They all had some antihistamine action,

but this action was less than in the experiments on the intestine and uterus. The action of neoantergan was more than 3 times that of benadryl

Rabbit's heart—In some of the earlier experiments the hearts did not beat, apparently because of deficient oxygenation. These experiments provided an opportunity for studying effects on the coronary flow uncomplicated by the secondary effects of changes in the beat which have been discussed by various authors (see Hammouda and Kinosita, 1926). The experiments may be compared with those of Wiggers (1909), who deliberately stopped the beat in order to avoid such complications.

Histamine (20 μ g) always decreased the flow Antergan (50 μ g) increased the flow slightly, and when given 1 min before histamine it abolished and sometimes reversed the effect of the latter drug. In one experiment 3277 RP (50 μ g) in creased the flow and caused a small diminution of the effect of histamine

When the heart was beating the antihistamine drugs depressed the amplitude of the beat to various degrees and they all decreased the stimulation due to histamine, but normally did not abolish it entirely. Neoantergan was more active than 3277 RP and more than 3 times as active as antistin. In a dose of 25 μ g it had a large effect against 4 μ g of histamine, but was almost inactive against 20 μ g Similar doses of benadryl abolished the response to 0.5 μ g of histamine, but only slightly depressed the response to 1 μ g

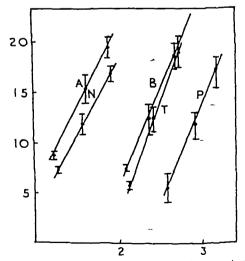


Fig 7—Frog's plexus anaesthesia Ordinates time to onset of anaesthesia in minutes Abscissae negative log molar concentration A = Antistin N = Neoantergan B = Benadryl T = 3277 RP P = Nupercaine Each point is the mean of 4-8 tests + the estimated standard error

Local anaesthesia

Halpern (1942) states that derivatives of the antergan series are potent local anaesthetics, but gives no details Benadryl (Friedlander and Feinberg, 1946) and antistin (Meyer and Bucher, 1946) have been shown to be local anaesthetics Using intracutaneous injections in guinea-pigs, Dews and Graham (1946) found that neoantergan was 31 times as potent as procaine, and Leavitt and Code (1947) found that benadryl was 25-6 times as active as procaine in human beings

Fig 7 shows the results of a comparison of four antihistamine drugs with nupercaine, by their effect on the frog's lumbar plexus. The time to the onset of anaesthesia is plotted against the logarithm of the concentration and the results fitted by straight lines. As judged by the concentrations needed to cause anaesthesia in 15 min, the relative molar activities of the drugs tested were as follows—nupercaine 100, 3277 RP 30, benadryl 26, neo-antergan 55, antistin 3

DISCUSSION

The experiments with guinea-pig ileum gave the best estimates of the relative activity of the various drugs against histamine (see Table) Neoantergan was the most active, 3277 RP was slightly less active, but its effect was slower in onset and lasted longer Antistin was much less active

Experiments on the uteri and isolated hearts were less complete but gave similar results. Whenever they were tested the above drugs showed antihistamine activity and the order of their activities was as given above.

The activity of the thiodiphenylamine drugs was discovered by Halpern and Ducrot (1946), who found that when 10-20 mg of 3277 RP was injected subcutaneously in guinea-pigs it protected them against the immediate effects of 1,400-1,500 lethal doses of histamine given intravenously 20 min later, while neoantergan only protected them against 100 lethal doses It was therefore surprising that 3277 RP was slightly less active than neoantergan on isolated organs. The meaning of this discrepancy is obscure, but may perhaps be related to the fact that the effects of 3277 RP are more The results described above are more prolonged easily reconcilable with those of Winter (1947), who found that neoantergan was more active than 3277 RP in antagonizing bronchospasm, contraction of the intestine and the lethal effect of small doses of histamine in guinea-pigs

Besides being the most active of these drugs against histamine, neoantergan is, so far, also the

most specific Benadryl and 3277 RP are both much more active than neoantergan against acetylcholine not only on the intestine but also on the Antistin has little action against acetylcholine on the intestine, but neoantergan has less The specificity of neoantergan was also shown in experiments with other agonists. In concentrations which suppressed the action of histamine it has little or no effect on the actions of potassium or nicotine on the guinea-pig's intestine or on the action of adrenaline on the rabbit's uterus Higher concentrations did, however, antagonize the actions of all the drugs used in experiments on the intestine, and according to Bovet and Walthert (1944) neoantergan also antagonizes the response of the rabbit's uterus to adrenaline These results probably explain the conclusions of Danielopolu et al (1941-5), who deny any spécificity to anter-They certainly used very large quantities of the drug, although the exact dose is not mentioned Schild (1947) also found that antihistamines in sufficient concentrations antagonize the actions of other drugs besides histamine Neoantergan is clearly the best of the drugs studied to use in specific tests for histamine, but it is useless if high concentrations are used Experiments of the type shown in Fig 4 are particularly suitable for tests of this kind, since they provide a quantitative comparison of the antagonism of the drug to histamine and to the unknown solution

The antihistamine drugs were found to depress the isolated heart and dilate the coronary vessels. They antagonized the actions of histamine on the force of the beat and on the coronaries, whether this was constrictor or dilator. The fact that this antihistamine action was comparatively feeble may perhaps be due to the conditions of administration, since the tissues were only exposed to the drugs for a very short time.

The local anaesthetic effects of these drugs were quite unrelated to their action against histamine, but may perhaps be related to their action against acetylcholine Benadryl and 3277 RP were, in fact, much more potent than antistin and neoantergan, both as local anaesthetics and as acetylcholine-antagonists. It would, however, be unwise to lay stress on these facts until the experiments have been extended to other drugs and to other methods of testing for local anaesthesia.

SUMMARY

1 The actions of a number of histamineantagonists on isolated organs have been compared in various ways

- 2 When tested by their power to antagonize histamine they were placed in the following order of descending activity—neoantergan, 3277 RP, benadryl, antistin, nupercaine
- 3 Neoantergan was the most specific of the drugs used Its action against histamine was greatest, and its action against acetylcholine was least Its action against nicotine, potassium, and adrenaline was much smaller than its action against histamine
- 4 Neoantergan is the best of these drugs to use in the identification of histamine in unknown solutions, but may give misleading results unless used in very low concentrations. A method of carrying out such tests is described
- 5 A method for the rough biological assay of neoantergan is described which involves the use of only about $0.002 \mu g$ of the drug per dose
- 6 The antihistamines tested depressed the beat of isolated hearts and increased the coronary flow
- 7 The activity of these drugs as local anaesthetics on the frog's lumbar plexus appeared to be more nearly related to their activity against acetylcholine than to their activity against histamine

These experiments were done during the tenure of a British Council scholarship

I am glad to express my thanks to Prof J H Gaddum for his hospitality and help, and to Dr H Adam for urinary extracts and the tracing shown in Fig 4

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NITROAKRIDIN 3582 A COMPOUND POSSESSING CHEMOTHERAPEUTIC ACTIVITY AGAINST THE VIRUSES OF PSITTACOSIS AND LYMPHOGRANULOMA VENEREUM

RY

E WESTON HURST

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(Received November 5 1947)

"Nitroakridin 3582" (Hochst) or 2,3-dimethoxy-6-nitro-9(3'-diethylamino-2'-hydroxypropyl)amino - acridine dihydrochloride

NH CH₂CHOH CH₃N(C₂H₆)₂ 2HCl

was developed by the Germans, who claimed that it possessed activity against rickettsiae Snyder, Hamilton, Fox, and Jackson (1946) found it to exert a beneficial effect on mice and embryonated eggs experimentally infected with murine or epidemic typhus, tsutsugamushi disease or Rocky Mountain spotted fever Green, Rasmussen, and Smadel (1946) observed a chemotherapeutic effect of the compound in embryonated eggs infected with the Lee strain of influenza B virus their experiments they mixed the compound with virus and inoculated the mixture into the allantoic sac of 11-day embryos In two experiments nitroakridin was injected an hour before virus After 2-5 days they estimated virus in the allantoic fluid by means of the haemagglutination technique Only 3 of 107 eggs receiving 1-10 M I D of virus together with 0.5 mg nitroakridin agglutinated red cells, and then to a lower titre than did infected control eggs Against 100 or more MID of virus the compound exerted a less striking effect. The addition of nitroakridin to known positive allantoic fluids did not influence their titres, nor did the drug inactivate virus at room temperature in 15 minutes, about 5 minutes longer than the time occupied in inoculating eggs after preparation of a mixture

This claim of therapeutic activity against one of the influenza viruses seemed sufficiently important to warrant investigation of the action of the compound against other viruses My colleague, Dr P Gaubert, very kindly prepared a sample of nitroakridin, with which we obtained no favourable effect on influenza in mice or on infections caused by four neurotropic viruses. Against psittacosis and lymphogranuloma venereum, however, the compound showed moderate activity

EXPERIMENTAL

In suitable chronic toxicity tests the maximal tolerated dose of nitroakridin in mice weighing 20 g was 0 25 mg daily, given intraperitoneally as a single dose dissolved in 0.5 c c sterile distilled water. In many experiments the animals were infected with a virus two hours after the second dose, variations from this procedure are indicated in the appropriate places. Dosing continued for a variable period according to the virus in use and the exact object of the particular experiment. Most six-day-old chick-embryos tolerated a single dose of 0.5 mg injected into the yolk-sac two hours after virus had been given by the same route

In comparative tests with other drugs we gave sulphonamides orally twice daily in doses of 5 mg per 20 g, penicillin (96 per cent penicillin-II) by intraperitoneal injection of 500 units four times daily at 90 a m, 12 45, 50 and 9 30 pm. During the night, animals receiving penicillin were offered the drug dissolved in sterile distilled water from sterilized drinking bottles.

RESULTS

(a) Influenza

Table I sets forth the scores of the pulmonary lesions in mice infected intranasally with the PR-8 strain of influenza virus A and killed on the 7th day. The results in mice treated with nitroakridin do not suggest a therapeutic effect of the compound

In the haemagglutination test nitroakridin did not inhibit agglutination of fowl erythrocytes by influenza virus, on the contrary, the stronger solutions themselves produced strong agglutination

TABLE I

EFFECT OF TREATMENT WITH NITROAKRIDIN ON THE PULMONARY LESIONS OF MICE INFECTED INTRANASALLY WITH INFLUENZA VIRUS

5 = death with specific lesions on or before the 7th day 4-0 = extent of pulmonary lesions recorded according to conventional practice

E	Number	Dilution	Pulmonary lesions				
Exp	of doses' of drug	of virus	Treated mice	Untreated mice			
1	7	10 ⁻⁵ 10 ⁻⁶ 10 ⁻⁷	4, 4, 3, 3, 3, 3, 3, 1, 0, 0 = 24 4, 3, 3, 2, 1, 1, 1, 1, 0, 0 = 16 1, 1, 1, 1, 0, 0, 0, 0, 0, 0 = 4	5, 5, 4, 4, 4, 3, 3, 3, 2, 1 = 34 4, 3, 3, 2, 2, 1, 1, 1, 0, 0 = 17 1, 1, 1, 1, 1, 0, 0, 0, 0, 0 = 5			
2	7	10-4 10-5 10-6	5, 5, 5, 5, 5, 5, 3, 2, 2, 0 = 37 3, 3, 3, 2, 2, 2, 1, 1, 1, 0 = 18 4, 3, 3, 2, 2, 1, 1, 1, 0, 0 = 17	5, 5, 5, 5, 5, 4, 4, 4, 3, 2 = 42 5, 5, 4, 3, 2, 2, 2, 1, 1, 0 = 25 3, 2, 2, 2, 2, 2, 1, 0, 0 = 16			
3	8	10-4 10-5 10-6	5, 5, 5, 5, 5, 5, 5, 5, 4, 4 = 48 5, 5, 4, 4, 4, 3, 3, 2, 0, 0 = 30 3, 2, 2, 2, 2, 2, 1, 1, 1, 0 = 16	5, 5, 5, 5, 5, 4, 4, 4, 4, 3 = 44 5, 5, 4, 4, 3, 3, 3, 2, 2, 0 = 31 4, 3, 3, 3, 2, 2, 2, 1, 0, 0 = 20			

(b) Equine encephalomyelitis, louping-ill, St Louis encephalitis and rabies

Table II shows the mortality in groups of 30 mice treated or untreated with nitroakridin and infected with one or other neurotropic virus. In Eastern equine encephalomyelitis the compound apparently prolonged the mean period of survival slightly, clinically we noticed that several animals lingered on in a moribund state, an experience unusual with this infection. In louping-ill the compound produced a heavier mortality, which statistically was almost significant, and the average period of survival was shorter than in controls. With the virus of St. Louis encephalitis deaths

were very much more numerous in treated animals, though the average period of survival appeared to be prolonged. In rabies the death-rate was slightly but not significantly greater than in control animals, the period of survival being unaltered

None of these results suggests that nitroakridin may be a useful therapeutic agent for the diseases in question

(c) Psittacosis

From the beginning it was clear that nitroakridin had a clear-cut beneficial effect on psittacosis. This effect was apparent not only from statistical consideration of the completed experiments, the

TABLE II

EFFECT OF TREATMENT WITH NITROAKRIDIN OF MICE INFECTED WITH NEUROTROPIC VIRUSES

			Mortality in groups of 30 mice					
Virus		Route of	T	reated m	ice	Untreated mice		
	Dose	inoculation	Number of doses of drug	Deaths	Mean period of survival in days*	Deaths	Mean period of survival in days*	
Equine encephalomyeli- tis (Eastern strain)	1000 cerebral LD50	ıntramuscular	8	10	62	9	5 0	
Louping-ill	1000 cerebral LD50	ıntramuscular	8	16	12 9	9	14 5	
St Louis encephalitis	1 cerebral LD50	ıntracerebral	14	25	18 0	8	14 9	
Rabies (virus-fixe)	1000 cerebral LD50	ıntramuscular	14	25	15 1	19	14.9	

^{*} From time of inoculating virus

TABLE III

EFFECT OF TREATMENT WITH NITROAKRIDIN OF MICE INFECTED WITH PSITTACOSIS

			i -							-
					M	Iortality ir	groups	of 30 mice		
-	Ne .	Route of Day			Tı	reated mic	e	-	Untrea	ited mice
Exp	Dilution of virus	inoculation of virus	exp ended	Number of doses of drug	l imic	of first of drug	Deaths	Mean period of survival in days*	Deaths	Mean period of survival in days*
1	10 ^{-6 8}	intraperitoneal	22	8	26 hr be	fore virus	15	12 0	28	8 6
2	10-7 0	intraperitoneal	26 26	8 14	26 6-	fore virus	11 2	12 0 13 0	27 28	9 3 9 5
`3	10-7 0	intranasal intraperitoneal ",	28 28 28 28	21 15 12 10	26 1-		20 16 22 24	15 2 11 9 11 9 9 5	25 29	15 2 8 6
4	10 ⁻⁷ ⁵ =5 LD50 10 ⁻⁷ ⁰ =10 LD50 10 ⁻⁸ ⁰ =1000 LD50 10 ⁻⁷ ⁰ =10 LD50	intraperitoneal	42 42 42 42	13 13 13 13	4 hr aft 4 hr , 4 hr , 52 hr ,	, ,, , ,,	13 13 16 23	14 3 21 4 12 2 13 5	28 28	7 1 7 2
5	10 ⁻⁷ ⁶ =5 LD50 10 ⁻⁷ ⁰ =10 LD50 10 ⁻⁶ ⁰ =1000 LD50 10 ⁻⁷ ⁰ =10 LD50	intraperitoneal	36 36 36 36	13 13 13 13	4 hr aft 4 hr , 4 hr , 52 hr ,	, ,,	11 8 19 12	19 1 21 0 17 9 19 4	27 29	15 4 15 4
6	10 ^{-7 0} 10 ^{-5 0} 10 ^{-4 0}	intraperitoneal ,, ,,	40 40 40	14 14 14	26 hr bef 26 hr , 26 hr ,	, ,,	26 19 27	19 7 11 3 10 3	26 30 26	11 5 7 9 7 5

^{*} From time of moculating virus

casual visitor to the isolation-unit, at a time when untreated animals were dying, might see one cage of extremely sick (control) mice, and another containing more numerous and more active animals of which few or none were obviously ailing Table III illustrates a number of points regarding this therapeutic action

Experiment 1 demonstrated the decreased mortality and the longer survival time of treated as compared with untreated mice inoculated intraperitoneally

Experiment 2 showed that while comparable control groups gave remarkably uniform results, extension of the period of treatment from 6 to 12 days after infection (8 to 14 doses including those before infection) greatly enhanced the therapeutic effect

Experiment 3 failed to demonstrate an effect of similar magnitude when virus was instilled intranasally. Whereas when virus was injected intraperitoneally nitroakridin reduced mortality considerably and increased mean-survival-time, more prolonged administration of the drug had less

effect upon the number of deaths and none on the mean-survival-time of mice inoculated intranasally Even so, a definite effect was apparent in additional groups of mice treated exactly as the others, except that they were killed on the 8th day after intranasal inoculation and the focal lesions in the lungs counted The foci in the control (untreated) group of 30 animals numbered 68, while those in the treated group of similar size numbered only 28 The other point established by Experiment 3 is that after intraperitoneal injection of virus delays in beginning treatment, of 48 and 96 hours respectively, resulted in a reduced favourable effect, even with the longer period of delay, however, the tendency was towards more frequent or more prolonged survival than in control mice Confirmatory data on this point were obtained in Experiments 4 and 5 The partial benefit conferred by delayed treatment dispelled the suspicion that the greater efficacy of nitroakridin against intraperitoneally administered virus might be attributed to direct inactivation of the latter by drug still present in the abdominal cavity at the time of injection Further evidence pointing to this conclusion will be

considered later In *in vitro* experiments designed to elucidate its mode of action we found that concentrations of the drug of from 1 10,000 to 1 1,000 did not mactivate the virus fully within one hour, the mixtures still killed most of the mice moculated, though with the 1 1,000 concentration the incubation period of the disease was prolonged by one or two days

In Experiments 4-6 the period of observation of the animals was extended and the effect of various doses of virus studied If a large dose of virus be given (10-4), nitroakridin is apparently capable only of increasing survival-time without preventing ultimate death With a dilution of 105, and often 10⁻⁷, a considerable therapeutic effect is seen When the dose of virus is very small, however, there is a tendency towards rather poorer therapeutic results, particularly evident in Experiment 6 In that experiment, at the time when administration of the drug ceased most of the controls were dead while nearly all the treated mice were alive About a week later and apparently quite well many began to sicken, and the final mortality in this group was the same as among the controls This reactivation of the disease, which we have seen on several occasions, did not come wholly as a surprise, because we had previously discovered that treatment with nitroakridin does not eradicate virus from the mice, apparently healthy animals prevented from dying by such treatment carry fully virulent virus in a greatly enlarged spleen for at least a month. The fact that this reactivation tends to occur more often after small than after larger doses of virus would seem to merit further study, it recalls the recent observation of Peterson and Fox (1947) in tsutsugamushi disease treated with methylene blue that treatment may safely be dis-

TABLE IV
CHEMOTHERAPY OF PSITTACOSIS
The effect of various doses of nitroakridin
All treatments began 4 hours after intraperitoneal
injection of virus

Description	Number	Mortality in groups of 30 mice			
Dosage of Nitroakridin	of doses	of 30 mice Mean peri	Mean period of survival in days		
None	_	28	7 2		
0 25 mg in 0 5 c c water once daily	13	13	21 4		
0 125 mg in 0 25 c c water twice daily	26	21	10 8		
0 1 mg in 0 25 c c water once daily	13	26	10 2		
05 mg in 10 cc water once every three days	6	21	11 5		

continued sooner after a massive infecting dose than after a smaller one

Table IV shows the effect of varying the dosage of nitroakridin, the best results appear to follow a dose of 0.25 mg given once daily

In Table V the effect of giving virus and drug by different routes is shown. It appears that intravenous administration of the drug is more effective against intraperitoneally inoculated virus than is intraperitoneal medication. Medication by the latter route, however, clearly has an effect on both intraperitoneal and intravenous infections. These observations furnish additional support for the conclusions reached previously, viz, that the action of nitroakridin is not purely a local one in the peritoneal cavity.

TABLE V
CHEMOTHERAPY OF PSITTACOSIS

The effect of varying the route of administration of virus and of nitroakridin

Infecting dilution of virus 10-6 All treatments began 4 hours after injection of virus and continued daily for 16 doses Animals observed for 37 days

		Mortality in groups of 30 mice						
Inoculation of virus		Treate	ed mice	Untreated mice				
	Injection of drug	Deaths	Mean period of survival in days*	Deaths	Mean period of survival in days*			
intraperi toneal intraperi	intraperi toneal intraven-	20	12 9	28	118			
toneal	ous	8	176	·	_			
intraven- ous	intraperi toneal	17_	17 6	23	13 6			

^{*} From time of inoculating virus

Finally we made several comparisons of the acti vity of nitroakridin and other chemotherapeutic Table VI presents the results of one substances such experiment With our strain of psittacosis virus sulphadiazine and sulphamezathine consistently failed to prevent death and usually prolonged but slightly the mean period of survival, sometimes sulphadiazine appeared to be the more active, at other times sulphamezathine. In the experiment shown in Table VI, nitroakridin, while failing to influence the final mortality, increased considerably the mean period of survival It was, however, not nearly as efficacious as penicillin, though even after 18 days of therapy with this substance virus was not eradicated, many of the mice, apparently well throughout the period of therapy, began to sicken and die five to ten days after treatment was discontinued

TABLE VI CHEMOTHERAPY OF PSITTACOSIS

Comparison of nitroakridin with other chemotherapeutic substances

Dosing began one hour after intraperitoneal injection

Dosing began one hour after intraperitoneal injection of virus and continued for 18 days Animals were observed for 35 days

Mortality in groups	of 30 mice	
	Deaths	Mean period of survival in days
Controls	26	8 3
Sulphadiazine	28	9 2
- (5 mg /20 g twice daily, orally) Sulphamezathine (5 mg /20 g twice daily, orally)	26	8 7
Nitroakridin	29	12 4
(0 25 mg/20 g once daily, 1 p) Penicillin (500 units/mouse 4 times daily, 1 p)	19	23 8
	ľ	ı

(d) Lymphogranuloma venereum*

Our observations with this virus supply the data for Table VII Virus inoculated into the volk-sac killed all untreated embryos within 8 days, and most by the 6th day A dose of 0.5 mg nitroakridin injected into the yolk-sac two hours after virus killed a number of embryos within the first 24 hours Of the remainder a few survived to the 20th day, while most of those dying did so at a later stage than the controls A dose of 0.25 mg per egg resulted in no toxic effects but all embryos succumbed to lymphogranuloma, albeit more slowly than the controls Thus, against lymphogranuloma in chick-embryos nitroakridin exhibits moderate chemotherapeutic activity

COMMENT

It is well known that the virus of lymphogranuloma venereum is susceptible to chemotherapeutic attack by many sulphonamides MacCallum and Findlay (1938), Findlay (1940), Jones, Rake, and McKee (1941), Rodaniche (1942), Felton, Hebb, and Oliphant (1943), van den Ende and Lush (1943) and others have dealt with various aspects of their action against the experimental disease in mice, and Meiklejohn, Wagner, and Beveridge (1946) in the chick-embryo The last authors also showed that penicillin inhibited growth of this virus in the yolk-sac

Reports of the activity of the commoner antibacterial agents against the psittacosis virus have not been unanimous Rudd and Burnet (1941) failed to demonstrate in mice a therapeutic action of several sulphonamides, though Wiseman. Meiklejohn, Lackman, Wagner, and Beveridge (1946) and Early and Morgan (1946b) found sulphadiazine to be effective in mice, and Meikleiohn. Wagner, and Beveridge (1946) and Early and Morgan (1946a) in eggs Heilman and Herrell (1944). Bedson and May (1945), Meiklejohn et al (1946), Wiseman et al (1946), and Early and Morgan (1946a and b), using mice, developing eggs, or tissuecultures, have all observed activity on the part of penicillin The last-named authors noted that the antibiotic was less effective against intranasal than against intravenous infection in mice, and ineffective against intracerebral inoculation, oral sulphadiazine was apparently less effective against intraperitoneal than against intravenous or respiratory infection

Mauer (1938) reported that trypaflavin exerted a beneficial action in mice infected with psittacosis With this exception the present appears to be the

TABLE VII

EFFECT OF NITROAKRIDIN ON CHICK-EMBRYOS INFECTED WITH LYMPHOGRANULOMA VENEREUM

		Results in groups of 15 eggs									
Exp Dose per egg mg	Dose		Trea	ited		Untreated					
	Non- specific deaths	Specific deaths	Survived	Mean period of survival in days†	Non- specific deaths	Specific deaths	Survived	Mean period of survival in days†			
1	0 50	4	8	3	99	0	15	0	6 5		
2	0 50	6	7	2	8 0	0	15	0	5 4		
3	0 25	0	15	0	7.6	0	15	0	4 0		

^{*} Alternatively known as lymphogranuloma inguinale and climatic bubo

first record of the activity of an acridine compound against viruses of the psittacosis-lymphogranuloma group

We have shown that nitroakridin 3582 (Hochst) is moderately active against the viruses of psittacosis in mice and lymphogranuloma venereum in developing chick-embryos Against our strain of psittacosis virus it appears more active than sulphadiazine or sulphamezathine (which indeed have only slight therapeutic properties) and much less active than penicillin It is less active against intranasal than against intraperitoneal or intravenous infections

Nearly all authors, including Rodaniche (1943), agree that treatment with current antibacterial agents does not sterilize infections with these viruses, and that despite clinical improvement or survival the animals continue to harbour fully virulent virus In our experiments we have conthis observation for psittacosis nitroakridin, we have further observed that after treatment for as long as 18 days with nitroakridin or penicillin apparently healthy survivors are apt, a week or more later, to develop symptoms of psittacosis and die

SUMMARY

Nitroakridin 3582 (Hochst) possesses moderate therapeutic activity against the viruses of psittacosis and lymphogranuloma venereum It is more active than sulphadiazine and sulphamezathine but less active than penicillin A week or more after the termination of a prolonged course of either nitroakridin or penicillin mice which have apparently been enabled successfully to withstand infection with psittacosis virus frequently develop symptoms and die This observation accords with the general experience that the common antibacterial-agents. while suppressing infections with viruses of the psittacosis-lymphogranuloma group, do not eradicate virus, which may still persist in a fully virulent

I am indebted to Prof S P Bedson for the strain of psittacosis virus used in this work (M O H 154)

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THE TOXIC PRINCIPLE OF COURBONIA VIRGATA ITS ISOLATION AND IDENTIFICATION AS A TETRAMETHYLAMMONIUM SALT

BY

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A tuberous root submitted for examination from the Southern Sudan as having been concerned in the death of two women and since identified at Kew as Courbonia virgata A Brongn, a member of the Capparidaceae, has been found to contain a toxic principle of an unusual nature

The highly toxic nature of the root was immediately confirmed by oral administration of aqueous extracts to rabbits, but preliminary examination for alkaloids yielded negative results and no evidence could be found for the presence of a glucoside Purification of the aqueous extract by treatment with basic lead acetate was found, by toxicity tests, to leave the toxic principle in solution, and it was observed that the purified solution, after removal of excess lead with hydrogen sulphide, gave an immediate precipitate of wellformed dark green crystals with a metallic lustre on addition of Wagner's reagent (todine in potassium iodide) This crystalline precipitate contained free iodine, and was evidently the periodide of a On treatment with hot water it slowly dissolved with liberation of free iodine, which could ultimately be driven off completely After repeated evaporation of the resulting solution to dryness, baking at 100° C and taking up with water, followed by fractional crystallization, the iodide of the base was finally obtained in pure form as colourless, well-formed crystals Injection of an aqueous solution of this salt into rabbits and mice showed it to be highly toxic and to produce symptoms closely similar to those produced by the aqueous infusion of the root which had been The above treatment of the originally tested material had therefore successfully isolated, in the form of its iodide, the main toxic principle present in the root. Analysis of the periodide, re-formed from the purified iodide, has shown it to approximate to the formula BI I.

In conformity with the results obtained on the original root extract, it was shown that the base is not precipitated on alkalinization of a solution of the iodide with ammonia or caustic soda, and is not extracted from alkaline solution by the usual organic solvents, this indicated a base of an unusual type, most probably containing a quaternary nitrogen atom. Moreover the action of silver hydroxide on the iodide produced a strongly alkaline solution the conductivity of which was as high as that of a solution of caustic soda of equivalent concentration, and which, on evaporation to dryness, yielded a very hygroscopic solid

The lodide is very stable to heat. Up to about 400° C there was little sign of melting or sublimation, but on further heating an oily sublimate was produced An aqueous solution of the iodide was neutral, optically inactive, non-fluorescent, odourless, and appeared to be quite stable Analysis of the iodide was not possible here owing to lack of facilities for carrying out combustions Even a satisfactory estimation of nitrogen could not be carried out, as the compound either shows an extraordinary resistance in the-Kjeldahl analysis (even with a mercury-selenium catalyst) or loses nitrogen during the digestion. In consequence, a specimen of the salt was submitted to Dr Harold King of the National Institute for Medical Research, Hampstead, and I am deeply indebted to him for its identification as tetramethylammonium 10dide (Found C, 244, H, 60, N, 68, I, 626 Calc for C₄H₁₂NI C, 239, H, 60, N, 70, I, 63 1 per cent) Its picrate melted with decomposition at 315° C as did an authentic specimen, and a mixture of the two showed an identical behaviour

This is the first occasion on which a salt of tetramethylammonium hydroxide has been found in the vegetable kingdom Ackermann, Holtz, and Reinwein (1923) found it in the animal kingdom in a sea-anemone (Actinia equina) and gave it the name tetramine

As a qualitative test for the toxic principle of virgata (tetramine) the crystalline precipitate which it produces with Wagner's reagent can be used From warm, dilute solutions, either acid or neutral, the periodide rapidly separates as wellshaped rhombic crystals which are readily recognizable under the microscope By this means it has been shown that tetramine also occurs in the thick, scaly shoots of the superstructure of the plant, and in the leaves of the subsidiary shoots Its presence in the shoots and leaves of the specimen sent to Kew was confirmed in the same way Another specimen of leaves submitted from the South for identification purposes, and reputed to be of the same species as that involved in the fatalities. was identified at Kew as being Osyris compressa (Berg) A DC. absurdly like C virgata in the sterile state and could very easily be confused with it in the field " A purified extract of these leaves failed to give crystals of any sort with Wagner's reagent. From the incomplete data at present available it is estimated that the fresh root of C virgata contains about 0.2 per cent of the toxic base, and that about 0.25 g of the base, taken orally, has proved a lethal dose for adult human beings, death occurring within an hour The victims " did not vomit but appeared They threw their arms about, babbled incoherently, and were difficult to control" Subcutaneous injection into mice showed the lethal dose of the iodide, by this route, to be 0.5-1.0 mg per 25 g mouse, the observed symptoms being convulsive spasms, collapse, and death within 30 minutes Intravenous injection of 8 mg of the iodide into a rabbit caused death within two minutes rabbits some contraction of the pupils has been observed prior to death Daily sub-lethal doses (0.25 mg), during which 5 mg were administered subcutaneously, failed to confer any immunity on a mouse or to reveal any cumulative action of the poison, as, at the end of the series of injections, the mouse was badly shocked by injection of 1 mg and four days later was killed within two minutes by injection of 2 mg

From these results it is evident that C virgata is to be classified as a relatively highly toxic plant,

and from the toxicologist's point of view failure of the toxic principle to be extracted from alkaline solution by organic solvents is important. Unless its presence is suspected, and its properties known. it might easily be overlooked. After addition of 0 02 g of the iodide to 200 g of macerated liver the normal Stas-Otto procedure for recovery of alkaloids from such material vielded an aqueous solution of the toxic principle which, after further purification with basic lead acetate, gave an excellent positive result with Wagner's reagent. liver of the rabbit killed by intravenous injection of 8 mg of the iodide failed to give a definite positive result on application of this procedure The toxic properties of C virgata appear to be well known to the natives of the areas where the plant occurs (eg, it is said to be used for poisoning hyenas), so that its toxicological aspect, about which nothing was previously known, is likely to prove important in such regions. The plant is known to occur in northern Uganda, north-eastern Kenya, and parts of French Equatorial Africa, as well as in the Southern Sudan

A solution of tetramine iodide (1 part per 1,000) was tested for bacteriostatic action against Staphylococcus aureus, S typhosus, Proteus proteus, Ps pyocyaneus, and B subtilis, with negative results in each case. It has not been possible to have trypanocidal tests carried out here, but preliminary insecticidal tests show that it appears to be an effective stomach poison for house-flies

The worked described herein has involved much help and co-operation from a number of sources. The author therefore wishes to express grateful acknowledgment to the Keeper of the Herbarium and Library, Royal Botanic Gardens, Kew, for botanical identifications and information, to Drs E. S. Horgan, R. Kirk and M. H. Sati, and Mr. J. D. Lewis, of the Stack Medical Research Laboratories, for carrying out the biological tests, to the District Commissioner, Yirol, for providing material, to Mr. D. N. Grindley and Riad Eff. Mansour for much assistance on the chemical side, and to the Director, Sudan Medical Service, for permission to publish this communication

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INJECTIONS OF ADRENALINE AND NORADRENALINE, AND FURTHER STUDIES ON LIVER SYMPATHIN

BY

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It is fairly generally conceded that adrenaline injected into the portal vein of mammals causes a smaller rise of blood pressure than when injected into the saphenous, femoral, or jugular veins. This difference between the two modes of administration is less when a large amount of adrenaline is used and it has been shown many times (for references see Dawes, 1946) that it persists when the injection is made very slowly so that it cannot be attributed to retardation of the release of adrenaline into the general circulation. One explanation is that the liver destroys adrenaline, and that the rise of blood pressure after intraportal injection depends on the amount of adrenaline escaping into the general circulation.

The theory that the substance liberated on stimulation of the hepatic nerves in the cat is noradrenaline or some similar substance was put forward by Bacq (1934) and by Stehle and Ellsworth (1937) Greer, Pinkston, Baxter, and Brannon (1937, 1938) have also considered noradrenaline as a possible sympathetic mediator, and have compared the effects of hepatic nerve stimulation (liberating presumably pure sympathin E) with those of injections of adrenaline and noradrenaline in the same animal In a similar series of experiments, Gaddum and Goodwin (1947) found no evidence against the theory that liver sympathin is noradrenaline It was of interest, therefore, to observe the effects resulting from intraportal, intrajugular, and intra-arterial injections of l-adrenaline and dl-noradrenaline in the same animal. In the experiments described below, the activities of the two amines were compared quantitatively with one another by these routes with the object of ascertaining their site of action and fate It was hoped, also, to obtain further evidence of the similarity of action between injections of noradrenaline and hepatic nerve stimulation

Dawes (1946) showed that the pressor action of adrenaline injected into the portal vein of a spinal cat was both increased and prolonged by the simul-

taneous injection of aromatic diamidines and monoamidines, of aliphatic diguanidines, diamidines and monoamidines, and of guanidine itself. This work has been repeated for guanidine, and extended to include the effect of guanidine upon the injection of noradrenaline and hepatic nerve stimulation. Similar experiments with cocaine and ephedrine have also been included

Methods

In different experiments, spinal cats, cats anaesthetized with chloralose or urethane, and rabbits under urethane or pentobarbitone were used. Blood pressure records were taken from the carotid artery and injections of the drugs made into the femoral, jugular, or splenic veins, or into the external iliac artery so that the injected solution passed into the vessels of the opposite leg. In some experiments, injections were made into one of the two main splenic arteries. Contractions of the nictitating membrane were recorded isotonically, 7–10 days after denervation by removal of the superior cervical ganglion.

The uterus was fixed at its lower end and its movements recorded directly. Movements of the duodenum were recorded by tying off a segment which was filled with warm saline and connected by a cannula and rubber tubing to a bottle, the upper part of which contained air and was connected to a piston recorder. Solutions of l-adrenaline and dl-noradrenaline were prepared from the pure bases with N/100 HCl

The hepatic nerves were separated from the hepatic artery and divided centrally. They were stimulated by means of platinum electrodes and an ordinary coil. In most of the experiments with liver sympathin, cocaine hydrochloride (8 mg/kg) was given intramuscularly.

RESULTS

In confirmation of Dawes (1946), it was found that the relation between doses of adrenaline producing equal rises of blood pressure by the jugular and by the portal routes differed according to the amount injected The results shown in Table I are

TABLE I

COMPARISON OF EQUI-PRESSOR DÓSES OF *l*-ADRENALINE
AND *dl*-NORADRENALINE BY THE INTRAPORTAL AND
INTRAJUGULAR ROUTES IN CATS

Drug	Intra portal injection (A) µg	Intra- jugular injection (B) µg	Portal (A) Jugular (B)	Blood pressure rise ' mm Hg
l adrenaline	10 20 40 80	2 5 12 5 27 5	5 0 4 0 3 2 2 9	19 40 83 124
dl nor adrenaline	10 20 40 80	5 8 17 5 40	2 0 2 5 2 3 2 0	41 56 95 120

the mean values for 10 cats, it will be seen that when the dose of adrenaline injected into the portal vein is large, proportionately more reaches the general circulation through the hepatic veins and the ratio of portal to equi-pressor jugular dose decreases. On the other hand, the corresponding ratio with noradrenaline remains fairly constant. Why is this difference present? It is well known that both amines, being derivatives of phenylethylamine, are readily destroyed by the liver amine oxidase in vitro

A similar result was found in rabbits under urethane (Table II) although the ratios of portal dose to equi-pressor jugular dose for adrenaline have always been higher than in the cat. For the jugular route in rabbits, adrenaline was found to be a much more active pressor agent than noradrenaline, a fact clearly shown when all the results are plotted graphically (Fig. 1). Great similarity exists between equi-pressor doses of adrenaline and noradrenaline by jugular or splenic vein in the cat

TABLE II COMPARISON OF EQUI-PRESSOR DOSES OF l-adrenaline and dl-noradrenaline by the intraportal and intrajugular routes in rabbits

Drug	Intra- portal injection (A) µg	Intra- jugular injection (B) µg	Portal (A) Jugular (B)	Blood pressure rise mm Hg
<i>l</i> - adrenaline	25 50 100 200	2 4 -10 25	12 5 12 5 10 0 8 0	24 35 - 54 -80
dl nor adrenalme	25 50 100 200 300	10 20 33 75 100	2 5 2 5 3 0 2 7 3 0	17 24 35 48 60

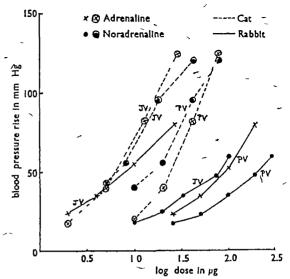


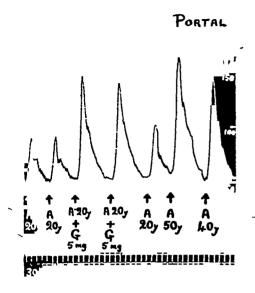
FIG 1—The effect of intrajugular (JV) and intraportal (PV) injections of l-adrenaline and dl-noradrenaline on the blood pressure rise in cats and rabbits. Note the great similarity between corresponding doses in the cat, and a wide margin between intrajugular doses of the two amines in the rabbit.

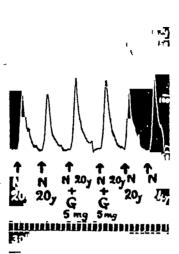
(the dotted lines), but a wide margin is indicated between equi-pressor doses by jugular vein Barger and Dale in 1910 pointed in the rabbit out that adrenaline and other similar methylaminobases had the property of exaggerating inhibitor as compared with motor effects, whereas the amino- and ethylamino- bases, including noradrenaline, possessed excitor with little or no inhibitory actions They also noted that a dose of ergotoxine sufficient to reverse the pressor effect of adrenaline in the spinal cat did not reverse that of noradrena-The observations reported here may be linked with the fact that in the rabbit, in contrast to the cat, there appears to be no sympathetic depressor component capable of being unmasked by blocking agents such as ergotoxine (Cannon and

TABLE III

RATIO OF DOSE OF *dl* NORADRENALINE PRODUCING A
RISE OF BLOOD PRESSURE OF 48 MM HG TO EQUI
ACTIVE DOSE OF *l*-ADRENALINE BY THE TWO ROUTES

Anımal	No	Preparation	Mean initial blood	Dose i idl nörad l adrei	renaline
	No Preparation	pressure mm Hg	intra- portal	ıntra Jugular	
Cat	5 2 3	Chloralose Urethane Spinal	118 65 62	0 8 1 5 1 8	0 5 1 3 1 5
Rabbit	2	Urethane	55	2 0	8 0





PORTAL

FIG 2—Spinal cat 2.5 kg Blood pressure record All injections into the portal circulation, 5 mg guanidine carbonate (G) greatly increases the pressor action of 20 μg adrenaline (A) but scarcely affects the pressor action of 20 μg noradrenaline (N)

Lyman, 1913), and in consequence the ratio of dose of noradrenaline to equi-pressor dose of adrenaline in rabbits is higher than that found in cats. On the other hand, the difference may be related to the initial blood pressure level. For example, as Table III shows, in cats under urethane with low blood pressures, noradrenaline is less active than adrenaline, whereas in cats under chloralose with high blood pressures, it is more active by both routes.

The potentiation of adrenaline injected into the portal circulation

Dawes showed that intraportal injection of amidines and guanidines into spinal cats increased the pressor action not only of intraportal adrenaline but also (and equally well) of the more active sympathomimetic amines, such as corbasil, epinine, and noradrenaline I have used guanidine only as the potentiating agent, and have compared its effect on adrenaline injections with that on noradrenaline injections Fig 2 shows the potentiation of 20 µg adrenaline, injected into the portal vein of a spinal cat, by 5 mg guanidine mixed in the same syringe On intrajugular injection, 5 mg guanidine itself had no significant effect on blood pressure and showed little or no potentiation of 5 μg of adrenaline There is therefore a striking difference between the action of guanidine on adrenaline injected by the portal vein and by the nugular vein Dawes suggested that the reduction of the inactivation of adrenaline in the liver by guanidine and amidine derivatives was not due to inhibition of mono-amine oxidase, but might be due to some interference with the uptake of adrenaline by the liver cells which prevented it reaching the enzyme

When these experiments were repeated with noradrenaline, no such potentiation was noted with intraportal doses of guanidine (Fig 2) or with intrajugular doses of guanidine The inactivation of intraportal doses of noradrenaline therefore is unaffected by the simultaneous injection of intraportal doses of guanidine Similar results were recorded in cats under chloralose or urethane and in rabbits under urethane, so that this fact is independent of anaesthetic and is not confined to one species Dawes showed that if a short interval of time was left between the injection of the guanidine and that of adrenaline the potentiation was less and that it disappeared altogether if the interval was ten minutes It is possible that the sloweracting noradrenaline may reach the enzyme when the guanidine is partially inactivated tively, the liver cells may inactivate noradrenaline slowly, especially as it may be that the substance liberated on stimulating the hepatic nerve in the cat is noradrenaline and not adrenaline (Bacq, 1934)

The action of guanidine on intra-arterial injection

It is well known that the injection of adrenaline into the femoral artery causes a much smaller rise of blood pressure than intrajugular or intrafemoral injection. Thus in spinal cats with a cannula in the external iliac artery (so that the injected solution passed into the vessels of the opposite leg), $12~\mu g$ adrenaline were required on intra-arterial injection to cause the same rise of blood pressure as $2~\mu g$ adrenaline by the jugular vein. As with

TABLE IV

COMPARISON OF EQUI-PRESSOR DOSES OF I-ADRENALINE AND dl-NORADRENALINE BY THE INTRA-ARTERIAL AND INTRAJUGULAR ROUTES IN SPINAL CATS

Cannulae in the external iliac artery and jugular vein

Drug	Intra- arterial injection (A) µg	Intra jugular injection (B) µg	Arterial (A) Jugular (B)	Blood pressure rise mm Hg
l adrenalme	12 25 40	2 5 16	6 0 5 0 2 5	28 49 76
dl nor adrenaline	10 15 40	2 5 16	5 0 3 0 2 5	29 43 62

the splenic vein injections, however, the ratio of equi-pressor doses by the two routes slowly decreased as the blood pressure rise increased (Table IV) When noradrenaline was injected similarly, ratio values slowly decreased, in contrast to fairly constant values after splenic vein injections. It follows that the inactivation processes of adrenaline and noradrenaline are very similar in the limb muscle vessels. Guanidine did not reduce the inactivation of either adrenaline or

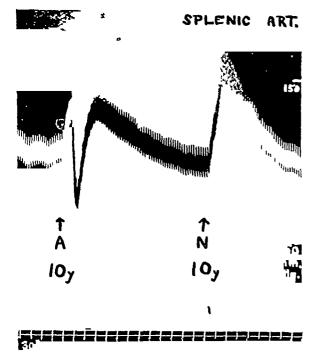
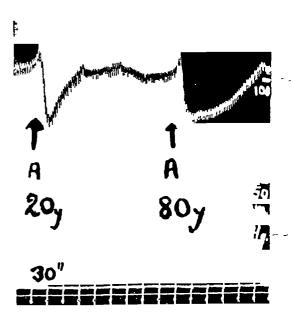


Fig. 3—The effect on the blood pressure of 10 μg adrenaline (A) and 10 μg noradrenaline (N) injected into the splenic artery of a chloralose cat

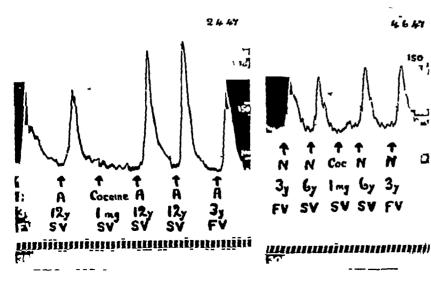


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Fig 4—Cat Chloralose 3.0 kg Blood pressure record The effect of adrenaline injected into the splenic artery. Note that the vasodepressor component of 20 μg is similar to that of 80 μg , but that the latter effect is more prolonged

noradrenaline when injected simultaneously into the femoral artery. In several animals even a depressed action was noted. If vasodilatation caused by guanidine was the principal cause of potentiation in the liver, then one would expect a potentiation on arterial injection. This does not occur and hence small doses of guanidine do not exert an effect on limb muscle vessels

When injected into the artery supplying the caudal end of the spleen, adrenaline in small doses caused a small rise followed by a large fall in blood pressure, quite similar to the effect of injecting crude cattle spleen extracts into the jugular vein of cats under chloralose (Euler, 1946a) depressor component was not completely abolished by intra-arterial doses of atropine, nor was it augmented by eserine Benadryl in a dose of 0 5 mg eliminated the depressor action of adrenaline at a time when the normal histamine response (10 µg) It suggests, therefore, that the was neutralized injection of adrenaline caused a liberation of histamine in the spleen, but more work must be completed before a definite conclusion can be reached



5 -- Chloralose Fig Blood pressure records After 1 cocame mg hydrochloride injected into the splenic vein (S V) 12 μg adrenaline intraportally is augmented, but not 3 µg adrenalme and 3 up noradrenaline intrafemorally (FV) or 6 μg noradrenaline intraportally

Noradrenaline, on the other hand, produced a pure rise of pressure at all dose levels (Fig. 3)

This effect of adrenaline on the blood pressure is very striking. It is possible, by using small doses intravenously, to obtain part of the effect (see Gaddum and Goodwin), but the dilatation following injection of 80 µg into the splenic artery is increased in length but not in depth, when compared with that after 20 µg adrenaline (Fig 4), whereas large doses intravenously produce pure vasoconstriction In addition, these effects are independent of the anaesthetic, since comparable results have been obtained in cats under ether or chloralose It is worthy of note that the latent period of noradrenaline injections is more than three times that of adrenaline by splenic artery, and this may indicate that the active adrenergic material in the spleen is not noradrenaline, which may have to be methylated or altered before it is effective similar longer latent period with noradrenaline than with adrenaline has already been noted (West, 1947a)

Intra-arterial doses of guanidine almost completely removed the depressor component of adrenaline, leaving the vasoconstrictor action, but had no effect on the noradrenaline response. When compared in the same cat under chloralose, equipressor responses were produced by 80 μ g of noradrenaline by splenic artery and 20 μ g by splenic vein (a ratio of 4 1), and by 300 μ g of adrenaline by splenic artery and 40 μ g by splenic vein (a ratio of 7 5 1). Thus noradrenaline is a much more potent pressor agent by splenic artery than adrenaline, and in addition relatively more adrenaline than noradrenaline is inactivated in the spleen

The action of cocaine and ephedrine

Experiments similar to those reported for guanidine were completed with 1 mg doses of cocaine hydrochloride and 100 to 150 μ g doses of ephedrine hydrochloride. Fig 5 shows the potentiation by intraportal doses of cocaine of intraportal but not intrafemoral adrenaline, and an absence of potentiation of intraportal noradrenaline. This supports the theory that the inactivation of noradrenaline in the liver is a slow process and is not affected by the simultaneous administration of substances such as guanidine and cocaine in small doses. As is well known, cocaine was found to enhance both adrenaline and noradrenaline responses when given by the same routes intrajugularly and intra-arterially (Table V). When

TABLE V

THE EFFECT OF INTRAVENOUS AND INTRA-ARTERIAL GUANIDINE AND COCAINE ON THE PRESSOR ACTIONS OF l-ADRENALINE AND dl-NORADRENALINE INJECTED BY SIMILAR ROUTES INTO CATS

A = adrenaline norA = noradrenaline + = potentiation 0 = no potentiation Dose of guanidine was 5 mg and that of cocaine was 1 mg

Route of	Potenti- ating	Jugular vein		Splenic vein		Iliac artery	
injection	agent	A	norA	4	norA	A	погА
Jugular vem	Guanidine Cocaine	0	0 +	0	0	0	0
Splenic vein	Guanidine Cocaine	0 0	0	++	0	0	0
Iliac artery	Guanidine Cocaine	0	0	0	0	Trace +	0

used in larger doses, all adrenaline and noradrenaline responses (including intraportal noradrenaline) were enhanced Ephedrine was an effective enhancing agent by all routes Thus, there are three types of potentiating agent used in this work (a)

TABLE VI

THE ACTIONS OF dl-noradrenaline and l-adrenaline, injected into the portal and jugular veins, on the blood pressure, denervated nicitating membrane, gut and pregnant uterus of two chloralose — cats — cocaine hydrochloride (8 mg/kg) was given intramuscularly

++= large effect += moderate effect 0= no effect

Route of injection	Drug	Dose µg	Mean blood pressure rise mm Hg	Nictitating membrane rise mm	Relaxation of gut	Contraction of uterus
Splenic vein	Adrenaline Noradrenaline	20 10	70 68	15 13	++	++++
Jugular vein	Adrenaline Noradrenaline	5 4	70 69	12 ′ 5	++	- ++

ephedrine, acting generally in the body and probably impeding or preventing enzyme inactivation processes when used in certain concentrations, (b) cocaine, acting in a manner similar to that of ephedrine but not enhancing intraportal doses of noradrenaline when itself given intraportally in small doses, and (c) guanidine, possibly acting directly on the liver cells preventing penetration of adrenaline to the amine oxidase, at the same time producing no potentiation of intraportal noradrenaline

The effect of adrenaline and noradrenaline on other organs in vivo

Records were taken of the actions of the two amines on the blood pressure, gut, uterus, and denervated nictitating membrane of two pregnant cats. The results of equi-pressor doses by the splenic and jugular veins are shown in Table VI It is of interest to note that noradrenaline by splenic

TABLE VII

THE ACTIONS OF dl-noradrenaline and l-adrenaline by the intraportal and intrajugular routes on the blood pressure, gut, and non-pregnant uterus of two spinal cats

Cocaine hydrochloride (8 mg /kg) was administered intramuscularly

++= large relaxation += definite but smaller relaxation 0= no effect

Route of injection	Drug	Dose µg	Mean blood pressure rise mm Hg	Relaxa tion of gut	Relaxa tion of uterus
Intra	Adrenaline Nor	10	62	++	++
portal	adrenaline	18	60	++	+
Intra-	Adrenaline	4	66	+	++
jugular	Nor adrenaline	6	66	slight	0

vein exerted its effects on the membrane, gut, and uterus, i e, it showed excitor and inhibitor actions, yet an equi-pressor dose by jugular vein gave very feeble actions. It is possible that part of the exogenous noradrenaline may be converted to adrenaline in the liver by N-methylation. Similar results were shown in two spinal non-pregnant cats, intraportal doses of noradrenaline being rather more active on the gut than on the uterus (Table VII). The fact that noradrenaline in moderate doses may inhibit the non-pregnant uterus and intestine in vivo confirms the results of previous workers (Gaddum and Goodwin).

Experiments with liver sympathin

Stimulation of the hepatic nerves in the cat almost invariably caused a rise of blood pressure, part of which was due to constriction of the hepa-The rise occurred even when a clamp on the artery itself raised the blood pressure a few mm Hg, so that the second effect was due to the liberation of sympathin (Gaddum and Goodwin) Fig 6 shows the effect of hepatic nerve stimulation before and immediately after the intraportal injection of 5 mg guanidine carbonate It will be seen that no potentiation of liver sympathin occurred In the same animal, adrenaline by the splenic vein was potentiated by guanidine but noradrenaline was not To overcome any damaging effect of guanidine on the liver cells, the adrenaline responses were completed after liver sympathin and noradrenaline effects had been shown, but the same result was obtained

In an animal which had not received cocaine, the effect of hepatic nerve stimulation was not potentiated by the simultaneous intraportal injection of cocaine hydrochloride (1 mg) Gaddum and Goodwin made it clear that cocaine was not essential for work with liver sympathin, since stimulation of the hepatic nerves caused a rise of

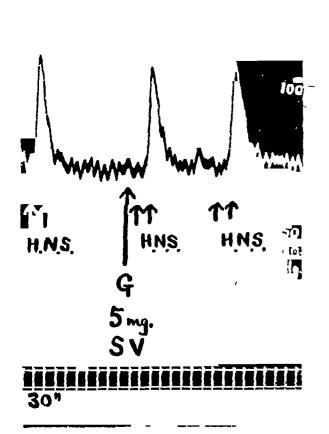


Fig 6—Cat 3 6 kg Chloralose Cocaine Blood pressure Stimulation of hepatic nerves for 30 sec (H N S) 5 mg guanidine carbonate injected into the splenic vein (S V) 15 sec before stimulation do not potentiate the pressor action

blood -pressure, contraction of the denervated nictitating membrane, and inhibition of the intestine before cocaine had been given They stated that whereas cocaine caused a definite increase of the effect on the nictitating membrane, it did not always affect the blood pressure rise The observations with guanidine and cocaine emphasize the great similarity in action between liver sympathin and nóradrenaline (Figs 2 and 6) Ephedrine potentiated the pressor effect of hepatic nerve stimulation in a feeble way The actions of liver sympathin on the nictitating membrane and pregnant uterus (excitor responses) and on the gut and non-pregnant uterus (inhibitor responses) agreed very well with those produced by equi-pressor doses of intraportal noradrenaline

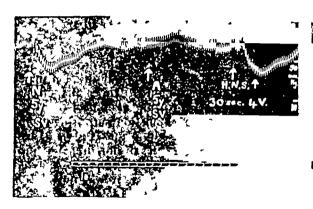
Cannon and Rosenblueth (1937) found that, after the administration of large doses of both ergotoxine (5 mg/kg) and cocaine to the same cat, the injection of adrenaline caused a pure fall of

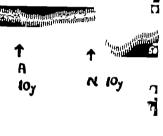
blood pressure but that stimulation of the hepatic nerves caused a rise We have observed similar results with ergotamine (3 mg/kg) By a chance observation, a large dose of dihydroergotamine (2 mg/kg) was given intraportally in a cat under chloralose This is about 10 times the normal adrenaline reversal dose, and the effect of subsequent hepatic nerve stimulation surprisingly produced a pure fall of blood pressure (Fig 7) Likewise, small intraportal doses of noradrenaline produced a fall while corresponding doses of adrenaline had little or no effect It was not always possible to repeat this result in later experiments For example, if the dihydroergotamine was given in small increments the normal adrenaline reversal was observed, but after hepatic nerve stimulation or noradrenaline administration a pure rise of blood pressure was produced Variable results were also produced if the dihydroergotamine was given by the intrajugular route In addition, intraarterial noradrenaline gave a rise and intra-arterial adrenaline a fall of blood pressure, whereas intrajugular noradrenaline gave a very small depressor These results only refer to cats under chloralose, and it is hoped to extend this work in order to see if the phenomenon occurs with other anaesthetics It is of interest to note that dihydroeigotamine does not always reverse the adrenaline response in dogs (Orth and Ritchie, 1947)

In two cats, the effects of stimulating the splenic nerve (dissected out in a manner similar to that used for the hepatic nerve) have been recorded So far, stimulation has produced a pure rise of blood pressure, no depressor component being observed, the rise although usually small was not potentiated by intra-arterial cocaine or guanidine

DISCUSSION

For small blood pressure rises in cats, noradrenaline was more active than adrenaline by both the jugular and the splenic routes As the pressure rise increased, so the sensitivity of the animal increased for adrenaline but decreased for noradrenaline Ratios of equi-pressor doses of the two amines were thus variable, and this observation may help to explain why such ratios have not been consistent in the past For example, Crimson and Tainter (1938, 1939) quoted many values for intrafemoral injection ranging from 05 to 12 For rabbits, noradrenaline was much less active than adrenaline by all routes studied explanations of these phenomena have been recorded (1) the rabbit appears to have no sympathetic depressor component comparable with that found in the cat, so that the response to adrenaline is pressor only in the rabbit, (2) the





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Fig 7—Cat Chloralose
Cocanie Dihydroergotamine (2 mg /kg.)
intraportally Note
that hepatic nerve
stimulation (H N S
30 sec) and intraportal noradrenaline
produce depressor
effects, whilst intraportal adrenaline
scarcely affects the
blood pressure

initial blood pressure levels partially regulate the ratio of equi-pressor doses of the two amines, for in low pressure animals (rabbits under urethane, spinal cats, and cats under urethane) the ratio is higher than 10, i.e., the pressor action of noradrenaline is less than that of adrenaline, whereas in high-pressure animals (cats under chloralose) it can be lower. In addition, it has been shown (West, 1947b) that adrenaline and not noradrenaline is a normal constituent of rabbit's blood. It is possible that adrenaline is the normal mediator liberated by adrenergic nerves in the rabbit, but that the mechanism is a little more complex in the cat

Guanidine on intraportal injection into cats and rabbits potentiated intraportal adrenaline potentiation was not shown by guanidine injected into the jugular or femoral veins or into the external iliac or splenic arteries. The effect is thus a local and transitory one on liver cells, presumably preventing the penetration of adrenaline (Dawes, 1946) Noradrenaline in equi-pressor intraportal doses was not potentiated, suggesting that the hepatic inactivation of this amine is not so rapid as with adrenaline Similarly, suitable intraportal doses of cocaine were found to potentiate intraportal adrenaline but not intraportal noradrenaline, whereas by all other routes studied both were potentiated Further, intraportal doses of noradrenaline produced effects on the pregnant cat uterus (excitor) and on the gut (inhibitor) when the equi-pressor dose by jugular vein was ineffective In addition, it has been shown that the inactivation processes for the two amines in the muscle vessels of the limb are similar in vivo since the ratios of equi-pressor doses by the arterial and femoral routes slowly decreased in both cases

The effects of intraportal noradrenaline have nearly always been similar to those of hepatic nerve stimulation. The simultaneous administration of cocaine and guanidine has not potentiated the pressor effect of stimulation. After large doses

of dihydroergotamine by splenic vein, both noradrenaline and hepatic nerve stimulation caused depressor actions when corresponding doses of adrenaline had no effect. In a biochemical study of this and other problems. Blaschko (1942) came to the conclusion on indirect evidence that adrena line is produced by N-methylation of noradrenaline which may be first formed in the body from tyrosine It is possible that-noradrenaline may be produced in the liver and may act as the adrenergic mediator in that organ, in which case the inactivation process of noradrenaline naturally would be suppressed A similar position occurs in the spleen, where injections of noradrenaline into the splenic artery and splenic nerve stimulation both produce vasoconstriction, whereas adrenaline injections result in the biphasic response with the marked depressor component. In addition, the ratio of equi-pressor doses of adrenaline by splenic artery and splenic vein is higher (75 1) than for corresponding doses of noradrenaline (4 1) Hence relatively more adrenaline is inactivated in the spleen than is noradrenaline, a result similar to that found for the-liver These observa tions with the liver and spleen may be linked up with the findings of Euler (1946a, b) that extracts of mammalian spleen, heart, liver, and sympathetic nerves contain a pressor substance with properties like those of noradrenaline or dihydroxy-norephe-In addition, he found large quantities of histamine in extracts of mammalian splenic nerves (Euler, 1947) This may account for the depressor component of the adrenaline response after injection into the splenic artery, since it is eliminated More recently, Bacq and Fischer by benadryl (1947) have reported that extracts of mammalian spleen contain only noradrenaline, extracts of human coronary nerves and arteries only adrenaline, but extracts of mammalian splenic nerves and sympathetic chains a mixture of noradrenaline and adrenaline Their interpretation of these facts is that in some tissues the synthesis of adrenaline

is stopped at the stage of noradrenaline, whilst in other tissues methylation occurs and adrenaline is formed They support Euler's suggestion that the term "sympathin" be used for a mixture of varying proportions of adrenaline and noradrenaline

SUMMARY

- 1. The pressor effects of dl-noradrenaline and l-adrenaline, injected into the jugular, femoral, and splenic veins and the splenic and external iliac arteries of cats and rabbits, have been examined
- 2 Adrenaline was less active by portal than by jugular vein, though the ratio value for equipressor doses by these routes decreased as the pressure rise increased. Noradrenaline was less active by portal than by jugular vein, but the ratio value remained constant.
- 3 When injected into the portal circulation, noradrenaline was not potentiated by the simultaneous administration of guanidine or cocaine whereas equi-pressor doses of adrenaline were enhanced. Noradrenaline therefore is not rapidly absorbed from the blood stream during its passage through the liver
- 4 Intra-arterial and intrajugular injections of adrenaline and noradrenaline were not potentiated by the simultaneous administration of intra-arterial or intrajugular guanidine, but both were enhanced by cocaine
- 5 Further similarity in the effects of hepatic nerve stimulation and intraportal injections of noradrenaline have been recorded Guanidine or cocaine in suitable intraportal doses, for example, do not potentiate the action of liver sympathin After large intraportal doses of dihydroergot-

amine, hepatic nerve stimulation and small intraportal doses of noradrenaline produced depressor responses, when corresponding doses of adrenaline were without effect

6 When injected into the artery supplying the caudal end of the spleen, adrenaline produced a depressor response, possibly due to the liberation of histamine Noradrenaline, on the other hand, produced a pure rise of blood pressure

My thanks are due to Mr K C Sparke for technical assistance

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CIRCULATORY PROPERTIES OF AMIDINE DERIVATIVES I. PRESSOR ANALOGUES OF METHYL ISOTHIOUREA

BY

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(Received March 6 1948)

The experiments to be described in this and the succeeding paper are concerned primarily with the circulatory properties of methyl isothiourea in relation to its physico-chemical constitution

Two considerations prompted such a study First, the chemical structure of methyl isothiourea is not at all complex, a diversity of related compounds were readily accessible Secondly, the pharmacological properties by which methyl isothiourea was known to be distinguished (Smirk, 1941, McGeorge, Sherif, and Smirk, 1942) seemed likely to permit the rapid testing of chemically A pressor action is the most related substances noteworthy of these properties, and, as the substance has been employed therapeutically for maintaining the blood pressure in spinal anaesthesia (Smirk and McGeorge, 1942), the possibility of finding other useful pressor agents was naturally a further inducement to the study of related substances

Screening experiments involving a wide range of compounds have already received brief comment (Fastier, 1944) Some of the points then raised are dealt with more fully in the present report, whose main purpose it is to outline difficulties in the way of a satisfactory explanation of the distribution of pressor activity among substances chemically related to methyl isothiourea

METHODS

Commercial samples of iminoazole (Kodak) and 2-aminopyridine (L Light) were used S,N-ethylene isothiourea hydrobromide was prepared from β -bromoethylamine hydrobromide and potassium thiocyanate, and S,N-propylene isothiourea hydrochloride by the action of warm concentrated hydrochloric acid on allyl thiourea. The effects of these N-substituted amidine derivatives upon the blood pressure of anaesthetized dogs and cats, the perfusion pressure

of pithed rat hind-quarters, and the tonus of isolated strips of rabbit intestine were recorded by methods used in earlier studies (Fastier and Smirk, 1943, 1947)

Methyl isothiourea methylsulphate and methylene di-isothiourea dihydriodide were synthesized by alkylating thiourea with dimethyl sulphate and methylene iodide respectively. Their higher homologues were obtained as hydrobromides by the method of Sprague and Johnson (1937) When no reference to the melt ing point of the recrystallized salt was found in the literature its composition was checked by a halide determination Attention was restricted (so far as this paper is concerned) to the blood pressure responses of anaesthetized animals to these other amidine derivatives, except with hexamethylene di isothiourea, the cardiac effects of the latter were studied (1) in anaesthetized cats by slipping a small glass cardiometer over the heart after the thorax had been opened medially and artificial respiration applied, (11) in rabbits by perfusing the isolated heart with Ringer-Locke solution by a technique described previ ously (Fastier and Smirk, 1943), and (iii) in anaesthe tized dogs by inserting a long glass cannula into the left jugular vein almost down to the auricle Clotting was prevented in the last type of experiment by filling the system connecting the cannula to a water manometer with a 5 per cent (w/v) solution of chlorazol fast pink, a little of which was run into the circula tion every few minutes The action of hexamethyl ene di-isothiourea on the arterial-blood pressure of decerebrated and pithed cats, and of anaesthetized and unanaesthetized grey rabbits (method of Grant and Rothschild, 1934), was also investigated, as were its effects on perfused rat hind-quarters and excised rabbit gut

RESULTS

Influence of chain-length upon pressor activity

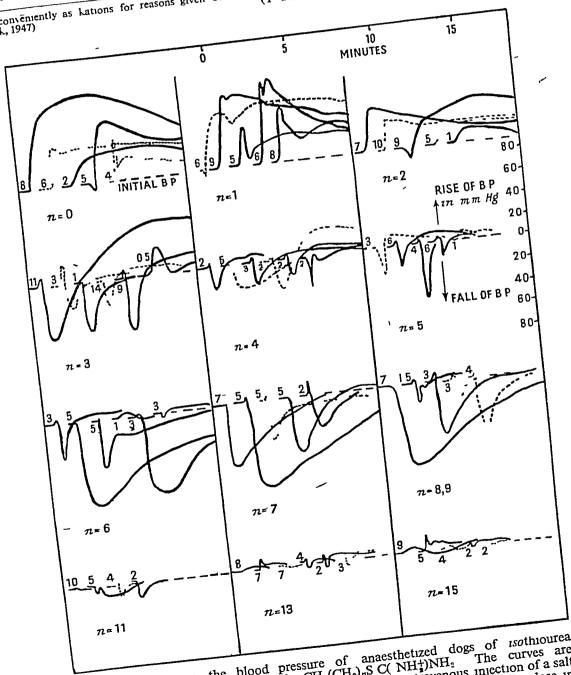
One conclusion reached on the basis of the above-mentioned screening experiments is that the pressor activity of basic amidine derivatives is affected adversely by an increase in the length of

To discover to what extent "ionic complexity, plays a part in determining activity, side-chains twelve homologues of methyl isothiourea of general formula* CH₂(CH₂)_nS C(NH₂+)NH₂ have been synthesized and their circulatory effects compared These alkyl isothioureas provide a series in which a gradual but ultimately considerable variation in

*Represented conveniently as Lations for reasons given before (Fastier and Smirk, 1947)

the physical properties of the kation is obtained with minimal deviation from the chemical structure of the prototype, methyl isothiourea (for which

The results illustrated in Fig 1 need little expla-It will be seen that lengthening the alkyl side-chain produces a notable alteration in the blood pressure response to a moderate initial dose nation (1-10 mg/kg) of the isothiourea salt



1—Effects on the blood pressure of anaesthetized dogs of isothiourea derivatives of general formula CH₃(CH₃)_nS C(NH⁺₂)NH₂. The curves are derivatives of kymograph tracings recorded after the intravenous injection of a salt derivatives of general formula Charles and Indiana, in courses are facsimiles of kymograph tracings recorded after the intravenous injection of a salt of one of the isothioureas, n refers to the particular one used. The dose in mg /kg is given alongside each curve vagotomy was not performed

increases, the ability to produce a large, well-maintained rise of blood pressure is soon lost. In contrast to methyl and ethyl isothiourea, the hexyl, heptyl, octyl, and nonyl homologues are predominantly depressor, when given under the same conditions, but the last three tested (n=11, 13, 15) are almost mert.

It should be added that the phenomenon of tachyphylaxis, already observed with various other amidine derivatives (Fastier and Smirk, 1943, 1947), was noticed with most of these isothiourea salts. Since the response to successive equal doses of a given isothiourea may change considerably in the course of an experiment and since treatment with one isothiourea often modifies at the same time the response to a related compound no less markedly, it is difficult to make a satisfactory comparison of the effects of several isothiourea salts in

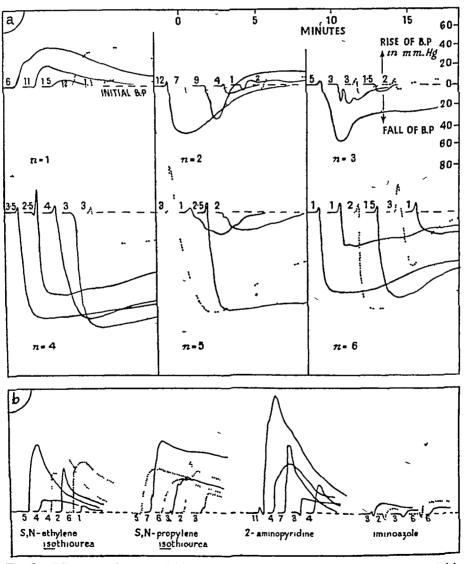


FIG 2—Effects on the arterial blood pressure of anaesthetized dogs and cats of (a) di-isothioureas of general formula H₂N(+H₂N) C S(CH₂)_nS C(NH₂+)NH₃, and (b) certain N-substituted amidine derivatives As in Fig 1, the curves are facsimiles of those recorded after the initial injection of the compound. The dose in mg/kg is appended to each curve. Experiments on cats are indicated by dotted curves In (a), n refers to the particular di-isothourea used.

the one animal For this reason, more reliance has been placed upon a comparison of the effects of initial doses of each isothiourea in fresh preparations

Depressor action of isothiourea derivatives, as exemplified by hexamethylene di-isothiourea dihydrobromide

Depressor activity was found to be even more striking in the higher members of the di-isothiourea

series of general formula

 $H_{2}N(^{+}H_{2}N) C S(CH_{2})_{n}S C(NH_{2}^{+})NH_{2}$

Only methylene di-isothiourea (n=1) possesses pres sor activity at all comparable with that of methyl isothiourea (Fig 2) The interest of the higher members tested (n=2-6) lies rather in the sustained falls of blood pressure they can elicit even when injected in fairly small amounts (1-5 mg/kg) Further experiments were therefore performed

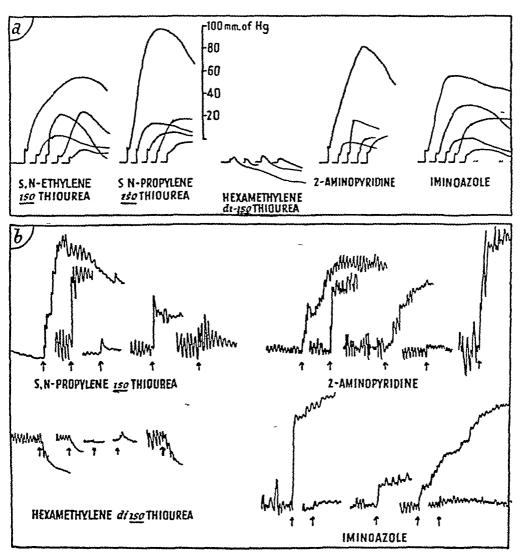


Fig. 3—Facsimiles of kymograph records showing effects of certain amidine derivatives on (a) pithed rat hind-quarters perfused with Ringer-Locke solution containing sufficient ergotoxine (1 200,000) to "reverse" the normal vasoconstrictor effect of adrenaline, and (b) excised rabbit intestine rendered insensitive to acetylcholine by atropinization

The N-substituted amidine derivatives tested constrict the perfused blood vessels and cause the contraction of the gut in the dose given (0.1 c c of the M/10 solution) even in the presence of these blocking agents. The "higher" amidine derivative hexamethylene di-isothiourea shows little resemblance to them in either preparation when injected in a corresponding dose (0.1 c c of the M/20 solution)

with the hexamethylene derivative in order to discover how the fall of blood pressure comes about

In all of 15 animals anaesthetized with sodium barbitone—7 dogs, 6 cats, and 2 rabbits—the intravenous injection of hexamethylene di-isothiourea dihydrobromide caused a persistent fall of blood pressure. The falls obtained in dogs with doses of 0.5–2.0 mg/kg ranged from 25–110 mm. Hg and lasted for from fifteen to upwards of sixty minutes (Fig. 2). Definite falls of blood pressure were produced in unanaesthetized as well as in anaesthetized grey rabbits.

Effects on cardiac output, so far as they could be discerned, did not suggest that the fall of blood pressure was due to cardiac depression thiourea did not reduce significantly the strength of contraction of isolated rabbit hearts when perfused in concentrations of up to 1 in 25,000, nor was there evidence of depression after its injection in 4 experiments on cats in which cardiometer records were obtained while the blood pressure was falling, rather the reverse. In dogs the venous pressure was lowered by some 10-30 mm H₂O in 2 out of 4 experiments after a temporary rise Electrocardiograms taken at the time did not reveal any noteworthy changes in rhythm The heart rate was usually found to be slowed when noted five to ten minutes after the injection of hexamethylene di-isothiourea, sometimes after an initial acceleration Vagotomy, which was performed in 7 out of 13 experiments, did not affect the fall of blood pressure

When a dilator action on blood vessels is thus indicated, it is difficult to prove that the action is a direct one Hexamethylene di-isothiourea was found to lower the blood pressure in decerebrated cats, but it did not have this effect when the animal was pithed, so long as the blood pressure remained at the low level to which it had fallen subsequent to the latter operation A vasodilator action was inconspicuous also in the pithed rat hind-quarters preparation except when the pressure had been raised beforehand by treatment with adrenaline or propionamidine The possibility therefore remains that its vasodilator action in the latter circumstance results from interaction with the vasoconstrictor drug, and not merely from the restoration of some degree of tonus to the blood vessels It was noted in this connexion that much smaller vasodilator responses to hexamethylene di-isothiourea were obtained when adrenalytic concentrations of ergotoxine were used in place of adrenaline or propionamidine to constrict the perfused rat blood vessels (Fig 3) A direct inhibitory action on smooth muscle was more definitely suggested by its effect on excised rabbit intestine, in a bath concentration of M/100,000 or upwards, hexa methylene di-isothiourea was found to depress the tonus and spontaneous movements of the strip (Fig 3), and to make it at the same time much less sensitive to the action of acetylcholine

Pressor effects of some N-substituted amidine derivatives

It had been noted on the basis of screening experiments (Fastier, 1944) that pressor activity is distributed fairly widely among amidine derivatives. Inasmuch as the structural relationship to methyl isothiourea (I) of pressor analogues like 2 aminopyridine (III) and iminoazole (IV) is far from close, the possibility must be considered, especially in view of the above results, that their pharmacological resemblance to it is merely superficial. The pressor actions of these particular amidine derivatives have therefore been analysed in some detail, along with those of S,N-ethylene isothiourea (II) and S,N-propylene isothiourea, N-substituted amidine derivatives whose structural relation ship to methyl isothiourea is more apparent

The rises of blood pressure produced in anaes thetized animals by the above bases in doses of the order of 1–10 mg/kg, though less persistent as a rule than those produced by methyl isothiourea under similar conditions, were fairly striking except in the case of iminoazole (Fig 2b). All four constricted perfused rat blood vessels about as strongly as methyl isothiourea (Fig 3a), their tonusincreasing effects on this preparation and on iso lated rabbit intestine standing in sharp contrast to those of hexamethylene di-isothiourea. Vasoconstrictor effects were not appreciably antagonized by treatment with ergotoxine. Moreover, atropine was usually ineffective in antagonizing their excitatory effects on gut (Fig 3b)

- DISCUSSION

So long as we remain ignorant of the exact site of action of methyl isothiourea we cannot say with certainty that any of its chemical relatives act in essentially the same manner, however likely this

may appear when various pharmacological effects are compared. It is tempting to assume that the pharmacological similarity to methyl isothiourea of its various pressor analogues has a chemical basis. Nevertheless such an assumption is reasonable only in so far as it can be shown that these pressor analogues have in common with methyl isothiourea either structural or physical features which are not shared with inactive relatives

The strong basicity of methyl isothiourea has already been stressed in this connexion (Fastier, 1944) Pressor activity has been found to be inconspicuous, if present at all, in imino-ethers and such other comparatively weakly basic chemical relatives as the ureas, carbamates, thiocarbamates, thioureas, and thiohydantoins which have the amide (V) or thioamide (VI) but not the structurally similar amidine (VII) group Even amongst the amidine derivatives used in these preliminary experiments, striking pressor activity was observed only with those that ionize freely

(V)
$$-C \stackrel{O}{\swarrow}_{NH_2}$$
 (VI) $-C \stackrel{S}{\swarrow}_{NH_2}$ (VII) $-C \stackrel{NH}{\swarrow}_{NH_2}$

But while possession of a strongly basic character therefore seems to be a necessary condition for pressor activity like that exhibited by methyl isothiourea, it has not proved possible to correlate what appears to be a fairly specific pharmacological action with possession of anything very specific from a structural viewpoint in the way of a "pharmacophoric" or "key" group In previous reports (Fastier and Smirk, 1943, 1947), it has been shown that pressor activity of apparently the same origin is exhibited not only by homologues like ethyl and isopropyl isothiourea but also by various other amidine derivatives of general formula $X \subset (NH_2+)NH_2-e g$, methyl iso-urea, ethyl isourea, propionamidine, methylguanidine, and asymdimethylguanidine, where $X=CH_xO_x$, $C_xH_xO_y$ C.H.-, CH.NH-, and (CH.)2N- respectively Salts of all these bases have been shown to cause, amongst other effects, constriction of perfused blood vessels, even in the presence of strongly adrenalytic concentrations of ergotoxine, and contraction of atropinized gut evidence which suggests that they owe their pressor activity in part at least to a capacity to constrict blood vessels by a direct action on their musculature In so far as 2-aminopyridine (III), iminoazole (IV), and other N-substituted amidine derivatives referred to above satisfy the same criteria, their pharmacological resemblance to methyl isothiourea is equally convincing

It will be noticed that these various musculotropic bases have nothing more in common from a structural viewpoint than the amidine group -C(NH-+)NH- How then are we to account for the finding (Fig 1) that merely lengthening the alkyl side-chain of methyl isothiourea seems to bring about a reversal of activity?

Presumably, if these pressor analogues of methyl isothiourea do have a fundamentally similar mode of action, we must give chief consideration to "physical" as distinct from purely structural attributes in trying to explain their pharmacological similarity on a chemical basis. The impression that their pressor activity is generally affected adversely by increasing the length of side-chains may therefore be highly significant, and this clue will be followed up in succeeding papers.

SUMMARY

- 1 In the series of *iso*thioureas of general formula $CH_3(CH_2)_nSC(NH)NH_2$, pressor activity is affected adversely by increasing the length of the side-chain (for n=0-9) Only the first three members are able to produce large, persistent rises of blood pressure in anaesthetized dogs.
- 2 Likewise in the di-isothiourea series of general formula $H_2N(HN)CS(CH_2)_nSC(NH)NH_2$, depressor effects rapidly become predominant as the series is ascended (n=0-6) The falls of blood pressure caused by the hexamethylene derivative when given in doses of 2-5 mg/kg are particularly large and long-lasting, they are brought about mainly, if not entirely, through vasodilatation
- 3 Although their structural relationship to methyl isothiourea is much less obvious than that of the above isothioureas, the N-substituted amidine derivatives 2-aminopyridine and iminoazole resemble it closely in their circulatory effects
- 4 Like S,N-ethylene and S,N-propylene isothiourea—and also various other amidine derivatives of low molecular weight examined previously (Fastier and Smirk, 1943, 1947)—2-aminopyridine and iminoazole constrict perfused-rat blood vessels even in the presence of strongly adrenalytic concentrations of ergotoxine, and cause the contraction of atropinized gut, thus they appear to act directly on the smooth muscle of blood vessels
- 5 It is concluded that if the pharmacological resemblance to methyl isothiourea of these various

pressor analogues has indeed a chemical basis, "physical" as distinct from purely structural attributes must play a large part in determining pressor activity in compounds of this type

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CIRCULATORY PROPERTIES OF AMIDINE DERIVATIVES II POTENTIATION OF THE VASOCONSTRICTOR ACTION OF ADRENALINE

BY

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A capacity to enhance the pressor action of adrenaline in anaesthetized animals is almost as conspicuous in methyl isothiourea* (I) and its nearer homologues as-their own pressor activity Both properties have been described before in some detail (McGeorge, Sherif, and Smirk, 1942, Fastier and Smirk, 1943), and evidence has been obtained which points to the blood vessel wall as the main site of action for pressor as well as potentiating effects

Various other amidine derivatives of fairly low molecular weight have since been found to produce closely corresponding changes in sensitivity to the pressor and vasoconstrictor actions of adrenaline Results obtained with derivatives of the type $X \subset (NH_2+)NH_2-eg$, ethyl iso-urea, propionamidine, and asym-dimethylguanidine, where $X=C_2H_3O_-$, C_2H_3- , and $(CH_3)_2N_-$ respectively—have already been reported (Fastier and Smirk, 1947) That even the presence of substituents in the amidine group itself does not invariably preclude this kind of activity will be shown below for such bases as S,N-ethylene isothiourea, 2-aminopyridine (II), and iminoazole (III)

Nevertheless "higher" amidine derivatives as a class seem to behave quite differently. So far from enhancing sensitivity to adrenaline when injected in moderately large doses, long-chain amidine deri-

vatives such as *n*-nonyl isothiourea (IV) and hexamethylene di-isothiourea (V) bring about no less emphatic desensitization. Their effects upon the blood pressure, discussed in the preceding paper (Fastier, 1948), appear equally anomalous at first sight. However, a more intensive study of the activity displayed by these compounds has indicated how such contrasting observations may be reconciled, as will also be shown below, an appreciation of the ambivalent character of typical amidine derivatives goes a long way towards explaining differences in effects upon sensitivity to adrenaline, outstanding though these may appear superficially

METHODS-

Experiments were performed upon dogs and cats anaesthetized with sodium barbitone and upon pithed rat hind-quarters perfused at a constant rate, as described in another paper (Fastier and Smirk, 1947) Ringer-Locke solution aerated with oxygen containing 5 per cent carbon dioxide was used for all perfusion experiments. Salts of the amidine derivatives were dissolved in Ringer-Locke solution to give the dilutions specified in the text, a little alkali being added as required when the resulting solution was acidic.

"Autosensitization"—an increasing sensitivity in the response of the preparation to adrenaline owing to intrinsic causes (vide Jang, 1940, and others whom he quotes)—was normally encountered at an early stage of each experiment. However, after some 30–60 min a stage was usually reached at which the response to adrenaline had become substantially constant, provided that 'cumulative" effects were also avoided by keeping the interval between successive injections to 6 or, on occasion, to as much as 9 or 12 min. Even so, the changes in sensitivity brought about by a given amidine derivative were sometimes difficult to assess because, as in the process of autosensitization, the height and the duration of the response to adrenaline were not always equally affected

^{*} Represented conveniently as a kation for reasons given before (Fastier and Smirk 1947)

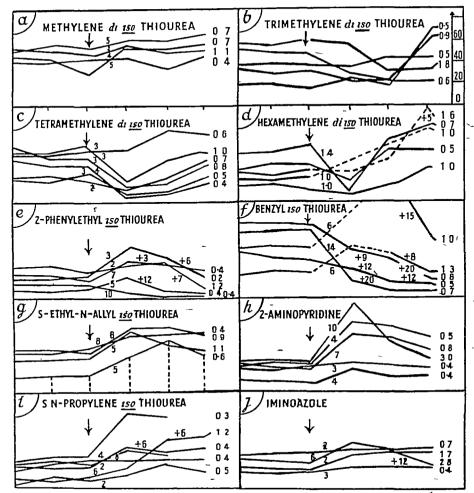


FIG 1—Effects of various amidine derivatives upon the sensitivity of anaesthetized dogs to the pressor action of adrenaline Each "sensitivity" curve was constructed as shown under (g), where the dotted ordinates are the rises of blood pressure caused by the injection of small doses of adrenaline at 6-minute intervals reduced to a common base line. The rise after the heavy arrow, which marks the point of injection of the amidine derivative (dose in mg/kg given alongside each curve), thus indicates an enhanced response to a fixed dose of adrenaline (dose given in \mu g/kg to the right of each curve). Dotted curves (d,f) refer to experiments where the dose of isothiourea was injected very slowly

The pressure changes due to the amidine derivative itself provided an additional complication. Plotting the heights of the initial pressure response to adrenaline was thought to provide as convenient and reliable an index as any of sensitizing and desensitizing effects, and this has been the procedure adopted in constructing Figs. 1 and 2

RESULTS

Experiments were first performed on anaesthetized dogs and cats. The results illustrated in Fig. 1 are typical of those obtained. They show that a variety of amidine derivatives are able to enhance the pressor action of adrenaline when their salts are injected in doses of the order of 1-10 mg/kg. They also show, however, that some amidine derivatives normally produce adrenalytic effects when tested under these conditions (Fig. 1c, d, f) and that others are far from constant in effect (Fig. 1b, e)

In order to see whether clear-cut results could be obtained with compounds of the latter type, two or three of them were given in widely graded doses. It so came to be noticed that while desensitization was produced eventually as the dosage was increased, sensitization was usually obtained at an earlier stage of the experiment. Some of the

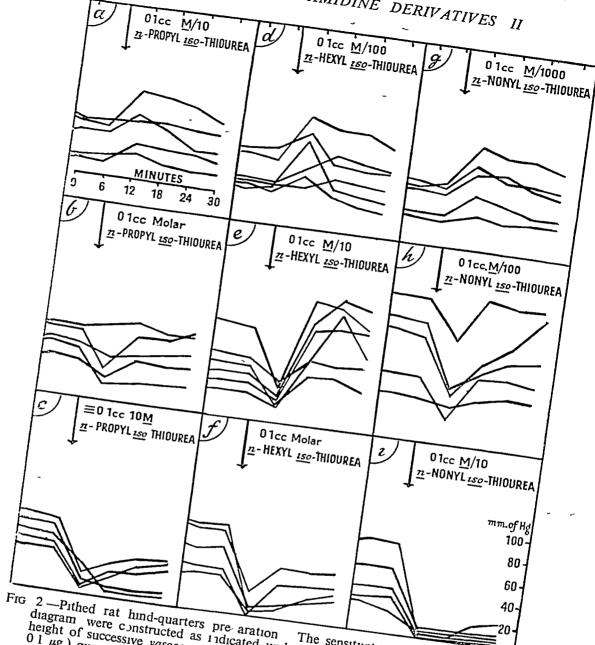


diagram were constructed as indicated under Fig. 1 but illustrate changes in the height of successive vasoconstrictor responses to a fixed dose of adrenaline (usually of the five experiments represented in neight of successive vasoconstrictor responses to a fixed dose of adrenaline (usually 0 1 µg) given at 6-minute intervals. Thus in the five experiments represented in adrenaline after the injection of a moderate dose of a proposition of a proposition of a moderate dose of a proposition of a moderate dose of a proposition of a proposition of a moderate dose of a proposition of a adrenaline after the injection of a moderate dose of *n*-propyl isothiourea compound can be seen to have produced equally emphatic desensitization in experiments in which larger doses were given (b,c)

Note that both sensitization and desensitization to adrenaline can be produced by higher than affine the affinet Note that both sensitization and desensitization to adrenaune can be produced also by higher homologues in appropriate doses (d,e,f, g,h,i) although the effect compound to another

"pure" adrenalytics were then examined these, it was discovered, were quite capable of producing sensitization to the pressor action of adrenaline, a dose which would have caused immediate desensitization if washed into the circulation all at once brought about no less emphatic sensitization, initially at any rate, when administered over a

period of 20-30 minutes (Fig. 1d, f) Consequently, the suspicion grew that the response to an amidine derivative might in general be influenced qualitatively as well as quantitatively by dosage following experiments were therefore carried out with a more typical compound, effects upon sensitivity to adrenaline being investigated on pithed rat

hind-quarters in preference to anaesthetized animals in order to simplify conditions as far as possible

Changes in the sensitivity of perfused rat blood vessels to the vasoconstrictor action of adrenaline induced by various doses of n-hexyl isothiourea hydrobromide

When small quantities (0.05-0.20 cc) of solutions varying in concentration from M/100,000 to 2M were injected one minute before the next dose of adrenaline was due, a threshold for sensitization was found with a concentration of the order of M/10,000 As the concentration of the isothiourea solution was increased, the sensitization produced increased in extent, becoming maximal for a concentration of the order of M/100 The vasoconstrictor response to adrenaline was frequently doubled with this dose (Fig. 2d)

With somewhat larger doses, however, the initial effect was a lesser degree of sensitization, and when the dosage was increased still further, actual desensitization resulted (Fig 2e, f) The desensitization was usually transient, in such instances (Fig 2e) a subsequent phase of sensitization followed regularly, which seems highly significant when it is recalled that the perfusion fluid does not circulate in the rat hind-quarters preparation as blood circulates in the intact animal, fresh Ringer solution is constantly being pumped into the dorsal aorta to renew that escaping from severed vessels If a drug is not held tenaciously by the tissues it must in consequence soon be washed out of the preparation Presumably, when such a dose as 01 cc of the M/10 solution is injected, the local (1 e, the "effective") concentration of n-hexyl isothiourea rises sufficiently high for it to exceed a threshold "desensitizing level" It would appear that this concentration cannot be maintained, however, and as it gradually diminishes, recovery takes place, to be followed by a period of sensitization Even with strongly adrenalytic doses, which might initially reduce the height of the vasoconstrictor response to adrenaline to one quarter or even one tenth of its former value (Fig 2f), this pattern was normally followed

The above interpretation of the form of the sensitivity curves shown in Fig 2d, e, f, was confirmed by experiments in which *n*-hexyl *iso*thiourea was administered by injecting it into the reservoir of Ringer-Locke solution instead of directly into the perfusion cannula. As but 15–20 c c of Ringer-Locke solution entered the preparation each minute, diluting the *iso*thiourea in some 200–400 c c of the perfusion fluid ensured that it could not rapidly achieve a high concentration in the tissues

even if the amount ultimately reaching the preparation was large Under these conditions it was found possible to demonstrate that desensitization is definitely preceded by sensitization when a large dose of isothiourea is given (as would be expected if the type of effect produced depends mainly upon "effective" concentration) Moreover, desensitization occurred the more rapidly the higher the concentration of *n*-hexyl isothiourea (M/20,000,M/5,000, M/2,500, M/1,000) or the faster its rate of perfusion Recovery followed the reverse pat-Thus on returning to plain Ringer-Locke solution after perfusing an M/5,000 solution of n-hexyl isothiourea, normal sensitivity was regained within a few minutes and followed by fairly longlasting sensitization Only in those experiments in which one of the stronger solutions had been perfused for a considerable time was sensitization not observed after changing back to ordinary Ringer-Locke solution, apparently because the dose had reached a toxic level, judged by the persistent loss of irritability of the preparation

Sensitizing and desensitizing effects of homologues of n-hexyl isothiourea

Nine other isothioureas of general formula $CH_3(CH_2)_nS$ $C(NH_2^+)NH_2$ were subsequently tested on the pithed rat hind-quarters preparation, viz, methyl, ethyl, n-propyl, n-butyl, n-amyl, n-heptyl, n-octyl, n-nonyl, and n-decyl isothioureas, for which n=0, 1, 2, 3, 4, 6, 7, 8, and 9 Their effects upon sensitivity to adrenaline all seemed to be influenced by dosage in essentially the same manner as those of n-hexyl isothiourea (for which n=5)

Lower homologues differ from the *n*-hexyl member mainly in that sensitization is obtained over a wider range of concentrations. The doses required for the demonstration of desensitization with *n*-propyl isothiourea (Fig 2c) or with methyl or ethyl isothiourea are definitely. "unpharmacological"

With higher homologues, on the other hand, adrenalytic activity was the predominant feature As shown in Fig 21, the desensitization produced by n-nonyl isothiourea in such a dose as 0 1 c c of the M/10 solution is intense. Nevertheless even n-nonyl isothiourea was found capable of producing the opposite effect when given in sufficiently small amount (Fig 2g), as were also n-heptyl, n-octyl, and n-decyl isothioureas.

Likewise with the anaesthetized dog, in which n-nonyl and n-decyl isothiourea seemed at first to be purely adrenalytic, it was found possible to demonstrate sensitization by using a very small amount for the first injection and gradually work-

ing up the dose Still higher members (n=11, 13, 15) gave little indication of activity, because of their insolubility they were not tested on the rat hind-quarters preparation. The lower members of the series in doses of the order of 1-10 mg/kg enhanced the pressor action of adrenaline in anaesthetized dogs, and this property extended well up the series (from n=0 to n=7)

The pithed rat hind-quarters technique is the better adapted, however, to showing the variation encountered during the ascent of the series with 0.1 cc of the M/100 solution as the fixed dose, a gradual increase was apparent in the degree of sensitization produced, a maximum being reached at about the heptyl derivative the same time the period of detectable activity increased from some 15-20 minutes to upwards of 30-40 minutes However, from the propyl derivative upwards, there was an increasing delay before maximum sensitization was observed The desensitization level was actually exceeded for a time with this dose of n-hexyl isothiourea and higher homologues, although in order to prove the point for the heptyl and octyl derivatives the first dose of adrenaline had to be injected as soon as 30 seconds later, desensitization was not observed in those experiments in which the interval was increased to 2 or 3 minutes Its transitory occurrence was demonstrated even with the hexyl derivative, however, by injecting the adrenaline in a steady stream instead of in doses given at regular intervals, and thus obtaining a continuous measure of sensitivity No such device was necessary with the highest homologues tested, the desensitization obtained with n-nonyl and n-decyl isothiourea was considerable and relatively persistent even with this small dose (Fig 2h)

Sensitizing and desensitizing effects of other amidine derivatives

With the di-isothioureas of general formula $H_2N(+H_2N)CS(CH_2)_nSC(-NH_2+)NH_2$, increasing the length of the polymethylene chain (n=1-6)was likewise found to enhance adrenalytic activity It has been shown above (Fig. 1c, d) that the tetramethylene and hexamethylene derivatives produce desensitization quite consistently in anaesthetized dogs in doses as low as 2-5 mg/kg hind-quarters preparation their adrenalytic activity was equally pronounced Sensitization of the perfused blood vessels was also obtained with most of these di-isothioureas, although it could not be demonstrated regularly with the tetramethylene and hexamethylene derivatives even when their salts were perfused in high dilution It was an inconstant phenomenon also with benzyl and

2-phenylethyl *iso*thiourea salts in the rat hindquarters preparation. That these particular adrenalytics were able under some conditions at least to cause sensitization as well as desensitization to adrenaline was obvious enough, however, from the results obtained with anaesthetized dogs (Fig. 1e, f) and cats

A much closer resemblance to lower homologues. like methyl and n-propyl isothiourea, was displayed by the N-substituted amidine derivatives S,Nethylene and S,N-propylene isothiourea, 2-aminopyridine (II), and iminoazole (III) produce sensitization in the rat hind-quarters preparation even when injected in fairly large amounts For the demonstration of desensitization, doses equivalent to 01 cc of an M-10M solution were required Much smaller doses sufficed, however, with bases like S-ethyl-N-allyl isothiourea and 2-aminoquinoline which, though allied structurally to the above N-substituted amidine derivatives, have considerably larger kations

DISCUSSION

Nearly all the amidine derivatives whose activity has been studied in detail have been found capable, like *n*-hexyl isothiourea, of producing either sensitization or desensitization to the vasoconstrictor action of adrenaline, the latter effect becoming predominant sooner or later when the dosage was increased Differences in their effects appear to be quantitative rather than qualitative, for while the effect of a fixed dose varies greatly from one compound to another, a distinctive pattern can be discerned none the less if the series of effects produced by a wide range of doses is made the basis of comparison, as indicated in Fig 2

Perhaps the strongest evidence in favour of this idea that both "lower" and "higher" amidine derivatives produce essentially similar sensitizing and desensitizing effects lies in the gradual transition from one characteristic type of behaviour to the other seen during the partial ascent of the two homologous series of isothioureas tested. One plausible objection may be raised, however. Why does the effect produced by a fixed dose alter so considerably during the ascent of a homologous series if all the homologues produce a given effect by the same mechanism?

As Ferguson (1939) has properly emphasized in connexion with toxicity data, we must distinguish between "chemical" and "physical" attributes in comparing the pharmacological activity of two drugs. The fact that one of the "higher" members of an isothiourea series is able to produce the same pharmacological effect (e.g., desensitiza-

tion to the vasoconstrictor action of adrenaline) as one of its lower homologues when it is given in a much smaller dose does not necessarily mean that 1 molecule of the higher homologue is as effective as, say, 10 or 100 molecules of the lower homologue at the site of action, it might well be that the distribution co-efficient of the lower homologue was relatively so unfavourable that 10 or possibly 100 times as many molecules of isothiourea had to be present in the enveloping medium (the "external phase") in order to maintain the same number of molecules as the higher homologue at the site of action (the "biophase")

To press the argument a little further, we might interpret the above results as follows "lower" and "higher" amidine derivatives are able to influence the sensitivity of blood vessels to adrenaline in a characteristic manner by virtue of a particular chemical configuration (the charged amidine "head" of the molecule) which enables them to react with the same receptors, but they differ quantitatively in their effects owing to variation in the physical properties of the molecule as a whole, which leads in turn to variation in their capacity to reach the appropriate receptors or remain in contact with them Evidence believed to favour this possibility will be presented in a later. paper

SUMMARY

1 It has been shown for the first ten *iso*thioureas (n=0-9) of general formula

CH₃(CH)_nSC(NH)NH₂

that either sensitization or desensitization to the vasoconstrictor action of adrenaline in the pithed rat hind-quarters preparation may be observed after their administration, according to the experimental conditions employed

2 Which effect is produced seems to depend mainly upon dosage Sensitization to a varying extent will occur so long as the local concentration of the *iso*thiourea falls within certain limits (which are characteristic of a given compound), but once

the hypothetical upper limit is exceeded, desensitization appears and becomes increasingly emphatic if the local concentration of the base rises still higher

- 3 Experiments with various other strongly basic amidine derivatives—e g, methylene di-isothiourea, hexamethylene di-isothiourea, S,N-propylene isothiourea, benzyl isothiourea, 2-aminopyridine, iminoazole—suggest that they too show a qualitative resemblance to the alkyl isothioureas in producing first sensitization and then desensitization to the vasoconstrictor action of adrenaline when they are given in increasing concentration
- 4 Adrenalytic effects are observed more especially with the higher members of a series—eg, n-octyl isothiourea, tetramethylene di-isothiourea, and their near homologues. The desensitization to the pressor action of adrenaline which they produce in anaesthetized dogs probably depends (like the sensitization obtained with smaller doses) in part if not entirely upon some process occurring in the blood vessels themselves, since desensitization to the vasoconstrictor action of adrenaline can be readily demonstrated with these-"higher" amidine derivatives in the pithed rat hind-quarters preparation
- 5 The influence of chemical structure upon this kind of activity is discussed

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DIAMIDINES AS ANTIBACTERIAL COMPOUNDS

BY

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Study of the diamidines was first directed to protozoal diseases, their usefulness in bacterial infections being discovered subsequently, some of our early results on propamidine were cited by Thrower and Valentine (1943), and its value in burns and surface infections has been confirmed Fuller (1942) showed that Gram-positive were more susceptible than Gram-negative organisms to aliphatic diamidines, and we sought for aromatic diamidines with greater activity against coli, proteus, and pyocyanea as well as against staphylo-A preliminary account of our findings has been given in conjunction with clinical trials on two of the new compounds (Wien, Harrison, and Freeman, 1948) Now, while penicillin deals with many infections it has little effect against Gram-negative bacteria, and although streptomycin may be of more value there is still a need for other active compounds we found certain diamidines which should be of value in local chemotherapy since our experimental studies have had clinical confirmation (Kohn and Cross, 1948)

Compounds—They conform to the general formula Am B X B Am in which Am represents an amidine group in the 4 or 4' position, B a benzene nucleus, and X a direct linkage, either an oxygen atom or an $-O(CH_2)_nO$ - group, where n is an integer from 1 to 10 Since all the derivatives described have substituents in the 2 or 2' position in the benzene nucleus, they may be referred to for convenience in an abbreviated form, e g, dibromopropamidine signifying 4 4' - diamidino - 2 2' dibromo-diphenoxypropane, and a list of some of those examined will be seen in Table I Their preparation and properties (Newbery and Berg, 1945) will be described elsewhere The isethionates of the compounds are readily soluble, forming colourless, neutral, and stable solutions, but they are precipitated in normal saline, isotonic solutions may be prepared by the addition of 5 per cent (w/v) dextrose

MATERIALS AND METHODS

Antibacterial activity

Organisms—These were derived mainly from the Lister Institute (for NCTC numbers see Table II), except for Staph aureus, strain No 19, which was obtained from a case of osteomyelitis, and strain No 27, which was obtained from a case of staphylococcal septicaemia

Media —Hartley's broth was employed, with the addition of 2 per cent (w/v) glucose for streptococci, a thioglycollate medium was used for clostridia and human defibrinated blood was used in slide-cell experiments for staphylococci

Methods—(1) Bacteriostatic activity was measured by means of the two-fold serial dilutional method a heavy inoculum, one drop of a 1 10 dilution of a 24-hour broth culture, was added to each tube, and the highest dilution completely inhibiting visible growth was recorded after incubation for 18 to 24 hours at 37° C these results are marked (a) in Table II Bacteriostatic activity in blood was determined by Fleming's slide-cell method, the staphylococcal inoculum being adjusted to give about 40 colonies in the control cell the highest effective dilution was read where 5 colonies or less were visible (Freeman, 1948)

(2) Bactericidal activity was determined by sub-inoculations on to trypsin-digest agar growth on agar after 24 hours' incubation at 37° C denoted lack of bactericidal activity. These results are marked (b) in Table II. The bactericidal properties were investigated also by observing the rate at which Staph aureus and B coli were killed from determinations of the minimal effective bactericidal concentrations at intervals up to 24 hours

Antifungal activity

Method —This consisted initially in incorporating serial dilutions of a compound in 2 per cent glucoseagar in small tubes infected with spores, which were then left at room temperature for 5 days. The highest dilution completely inhibiting growth was observed

More reproducible results were obtained by employing a ditch-plate method.

The petri dishes contained 15 ml of 2 per cent glucose-agar, and a ditch (3/8 in) was cut right across the diameter of each agar plate The ditches were then filled with 2 per cent glucose-agar containing solutions of the compounds to be tested, ten-fold dilutions were tested first and subsequently two-fold dilutions, after the approximate range of activity had been determined The plates were then dried for one hour at 37° C Inoculations were made with a platinum needle from ten-day-old glucose-agar slopes species of fungi were employed for each plate, one streak being made of each fungus, crossing the ditch at a right-angle and continuing to the opposite edge of the plate. The plates were incubated at 37° C for 5 days except for Hormodendron langerony, which grew best at room temperature Fungistatic activity

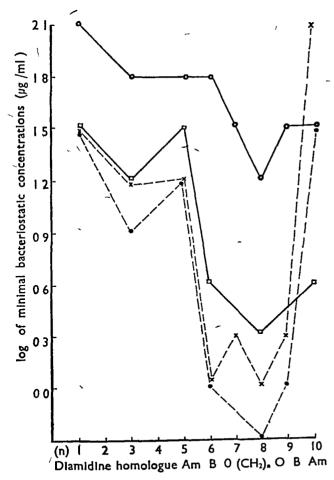


Fig 1—Bacteriostatic activities of homologous diphenoxyalkanes expressed as logarithms of minimal bacteriostatic concentrations. Note the increase in effect with increase in the length of the chain to a maximum at n = 8 o o B coli in broth B flexneri in broth A o B flexneri in broth B flexneri in broth B flexneri in broth B flexneri in broth B flexneri in broth B flexneri in broth B flexneri in broth B flexneri in broth B flexneri in broth B flexneri in broth

was determined by noting the highest dilution which caused complete inhibition of growth of the fungus on the ditch containing the compound A control was obtained by observing good growth of the streak on the agar which did not contain any compound Some zonal inhibition was seen at the edges of the ditch owing to diffusion of the compound into the untreated agar, but these zones were disregarded in the assessment of fungistatic activity

RESULTS

Bacteriostatic results on homologous diamidines -Several points are worth noting from the bacteriostatic results shown in Fig 1 Firstly, there was a graded increase in bacteriostatic activity against staphylococci with increase in the length of the chain to a maximum at n=6 to 9, followed by an abrupt decrease at n=10, against Gram negative bacteria the maximum was maintained from n=6 to 10 Secondly, activity was retained in the presence of blood Determination of the toxicities of these compounds on intravenous injection into mice showed an increase in toxicity in ascending the series from the methane (LD50, 30 mg/kg) to the decane (LD50, 5 mg/kg) derivative It should be noted that propamidine, though less active than its higher homologues, was also less toxic to phagocytes (vide infra)

Bacteriostatic results on homologous diguanidines—We found that homologous diguanidines displayed the same gradation in bacteriostatic activity as the diamidines against Gram-positive

TABLE I

BACTERIOSTATIC ACTIVITY OF HALOGENATED DIAMIDNES

Minimal concentrations in µg /ml for complete inhibition of growth

	Staph	aureus	B cols	Ps pyo- cyanea
Compound	in	in	ın	ın
	broth	blood	broth	blood
Phenamidine	128	64	256	512
Iodophenamidine	32	32	64	512
Stilbamidine	32	32	256	256
Iodostilbamidine	8	16	32	64
Bromopropamidine	4	2	32	256
Dibromopropamidine		4	4	32
Iodopentamidine	0 5	1 5	64	128
Diiodopentamidine	1	4	8	64
Iodohexamidine	0 5	1 8	16	8
Duodohexamidine	1		4	16

organisms Against Staph aureus in blood, in slide-cell experiments, the minimal concentrations inhibiting growth were as follows —4 4'-diguani-dinodiphenoxyethane, 32 μg /ml , propane homologue, 8 μg /ml , butane homologue, 4 μg /ml , pentane homologue, 2 μg /ml , heptane homologue, 4 μg /ml , and 256 μg /ml for the nonane and decane homologues respectively

Bacteriostatic and fungistatic results on halogenated diamidines—Table I gives the results obtained with some of the halogenated diamidines examined the diphenoxyalkanes were bacteriostatically more active than the stilbene compounds, and the diphenyl ethers were much less active. The halogenated diamidines were effective against certain pathogenic fungi, though not more so than the parent compounds, the degree of fungistatic action seemed to depend on the type of fungus, although some of the variation may have been due to the method employed

Since the diphenoxyalkanes showed promising

bacteriostatic activity, various substituted derivatives were examined, it was found that halogen in one or both benzene nuclei had a favourable effect, increasing bacteriostatic activity with little alteration in local toxicity to phagocytes. The mono-halogen derivatives were more active than the di-halogen derivatives against staphylococci, whereas the di-halogen derivatives were more active against Gram-negative bacteria.

Dibromopropamidine and iodohexamidine were found amongst the most effective of the compounds examined, and the results obtained with these and their parent compounds are shown in Table II For comparison, penicillin (pure sodium salt) was effective in inhibiting the growth of Staph aureus at a minimal concentration of 0.05 μ g/ml, and streptomycin was bacteriostatically effective at 8 μ g/ml against B coli, and at 4 μ g/ml against Ps pyocyanea

Bactericidal results on halogenated diamidines
—The bactericidal activity (results marked (b) in

TABLE II

ACTIVITY OF PROPAMIDINE, DIBROMOPROPAMIDINE, HEXAMIDINE, AND IODOHEXAMIDINE AGAINST VARIOUS

BACTERIA AND FUNGI

(a) = bacteriostatic activity. (b) = bactericidal activity. (f) = fungistatic activity. Minimal concentrations

(a) = bacteriostatic activity, (b) = bactericidal activity, (f) = fungistatic activity Minimal concentrations in μg /ml for complete inhibition of growth

- Organism	NCTC* strain No	Propamidine	Dibromo- propamidine	Hexamidine	Iodo- hexamidine
Strep pyogenes Strep viridans Staph aureus ,, ,, (in 10% serum) ,, ,, (in blood) ,, ,, (in blood) Ps pyocyanea ,, ,, (in 10% serum) Proteus vulgaris B coli ,, ,, (in 10% serum) B flexneri B enteritidis B typhi-murium Cl welchii Cl histolyticum Actinomyces kimberi ,, madurae ,, hominis Geotrichum dermatitidis Trichophyton tonsurans Hormodendron langeronii	2432 3165 †19 19 19 19 1999 3156 •4144 4144 4835 4444 2110 273 2915 4583 3255 4525 2787 2520 2893	(a) 4 (a) 4 (a) 8 (b) 16 (a) 16 (a) 16 (a) 256 (b) 256 (a) 256 (a) 256 (a) 128 (b) 256 (a) 64 (b) 128 (a) 128 (a) 32 (a) 256 (a) 32 (a) 256 (a) 32 (a) 256 (b) 100 (f) 100 (f) 100 (f) 100 (f) 100 (f) 100 (f) 100 (f) 100 (f) 25 (f) 100	1 2 1 4 4 4 16 32 64 128 128 256 4 32 8 8 64 64 512 256 10 50 1000 200 200 25 500	0 5 1 1 8 1 1 4 16 32 32 128 256 64 64 64 64 64 256 20 100 10 20 200 20	0 5 0 5 0 5 0 5 4 1 1 2 8 32 32 128 256 16 32 64 16 16 256 20 200 10 20 200 100

^{*} N C T C (National Collection of Type Cultures)

[†] Laboratory strain number

Media —Hartley's broth medium was used for bacteriostatic tests except where indicated, for streptococci 2 per cent (w/v) glucose was added, and for clostridia a thioglycollate medium was employed Trypsin-digest agar was used for bactericidal tests and 2 per cent glucose agar for fungistatic tests

TABLE III

ACQUIRED DRUG-RESISTANCE in vitro

Bacteriostatic activities minimal concentrations in μg /ml for complete inhibition of growth

Compound	Organism	Bacteriosta Initial	tic activities Maximal	No times increase in resistance	No sub- cultures	
Dibromopropamidine Propamidine 5-aminoacridine Penicillin	Strep pyogenes Staph aureus ,, ,,	1 8 8 0 03 1 u /ml	128 128 32 1400 1 u /ml	128 16 4 46,666	24 - 10 - 4 - 40	

Table II) of the halogenated diamidines was somewhat less (four to eight times) than their bacteriostatic activity. From determinations of the rate at which Staph aureus and B coli were killed by various concentrations of the compounds it was found that the maximum bactericidal effect was exerted fairly rapidly, within six to ten hours

Effect of pH

We confirmed Elson's (1945) observation that the bacteriostatic action of diamidines was decreased in an acid medium and increased in an alkaline medium. At pH 63, 68, and 76 the minimal effective concentrations of dibromopropamidine against staphylococci in blood were 64 μ g/ml, 4 μ g/ml, and 1 μ g/ml, respectively, and of propamidine 64 μ g/ml, 16 μ g/ml, and 4 μ g/ml respectively

Since the diamidines inhibit the respiration of bacteria (vide infra), further evidence was obtained by measuring the oxygen uptake of B coli in phosphate buffer at different pH values, in Warburg vessels, with various substrates initial incubation (37° C) with the diamidine for 15 minutes the percentage inhibition of respiration was measured at 10-minute intervals for 2 hours The manometer cups contained diamidine solution (0 00012 M), substrate solution (0 01 M), 0 5 ml washed suspension of B coli containing approximately 10° organisms per ml, phosphate buffer (0.033 M), and 0.2 ml of 6 per cent (w/v) potassium hydroxide to absorb carbon dioxide, the volume was adjusted to 3 ml with saline controls did not contain any diamidine sodium lactate as substrate, at pH 56, 68, and 78, the percentage inhibitions by propamidine after 2 hours were 10, 35, and 72 respectively, for dibromopropamidine they were 0, 51, and 82, for hexamidine 0, 26, and 77, and for iodohexamidine 29, 50, and 87 Similar results were obtained with alanine, glucose, and sodium acetate as substrates

It was evident that acidity decreased and alkalimity increased the inhibitory effect.

Acquired drug-resistance

Resistant-bacteria, whether naturally occurring or developed during treatment with chemical sub stances, are well recognized and may make selective treatment necessary Accordingly, serial dilutions of the compounds in broth (2 per cent glucose broth for streptococci) were infected with the organisms (various strains of staphylococci and streptococci isolated from wounds were used), and after incubation at 37° C for 24 to 48 hours the contents of the tube with the highest concentration of a compound showing growth was used for infec-The organisms were thus ting a further series trained by repeated subcultivation to increasing concentrations of the compounds, and the results in Table III show the degrees of resistance attained The resistance acquired by the various organisms was permanent and fairly readily induced Crossresistance experiments were also carried out, and these results are shown in Table IV The following conclusions can be drawn (1) staphylococci resistant to penicillin or to 5-aminoacridine were susceptible to diamidines, (2) staphylococci and streptococci resistant to one diamidine were resistant also to other diamidines, -(3) staphylococci resistant to diamidines were not resistant to peni-Treatment with dicillin or 5-aminoacridine amidines in the human subject might, but would not necessarily, produce similar resistant strains We encountered some strains of pyocyanea from wounds which, although initially resistant to diamidines, readily lost this resistance when kept for only a week in vitro on a drug-free culture medium, showing that the resistance, in this instance, was It should be noted that in our not permanent experiments the resistance produced by penicillin was much greater than that described by McIntosh and Selbie (1943)

TABLE IV

CROSS-RESISTANCE OF INDUCED DRUG-RESISTANT STRAINS OF Staph aureus and Strep pyogenes

Figures indicate bacteriostatic activities minimal concentrations in μg /ml for complete inhibition of growth

Organism	Organism Strep pyogenes		Staph aureus	Staph aureus	Strep pyogenes	Staph aureus
Dibromo- propamidine resistant		Propamidine resistant	Penicillin resistant	5-amino- acridine resistant	Original unmodified strain	Original unmodified strain
Dibromopropamidine Propamidine Hexamidine 5-aminoacridine Penicillin	128 256 32 8 0 1 1 u /ml	8 128 8 8 8 0 01 1 u /ml	1 4 0 5 8 1400 1 u /ml	1 16 1 32 0 01 1 u /ml	1 4 0 5 4 0 03 1 u /ml	1 8 1 8 0 03 1 u /ml

Toxicity and local tolerance (Table V)

- (a) Systemic toxicity for mice—The LD50 in mice was determined for both the intravenous and subcutaneous routes symptoms were similar to those previously described for other diamidines (Wien, 1943) Both dibromopropamidine and iodohexamidine had a depressor effect in the chloralose cat, dibromopropamidine increased—while iodohexamidine decreased the tone and movements of the isolated rabbit ileum
- (b) Toxicity to human leucocytes—Killed staphylococci were added for 30 minutes to citrated human blood previously in contact with diamidine solution for 3 hours at 37° C. After lightly centrifuging the blood in capillary tubes, leucocyte films were prepared from the upper surface of the cellular deposit and stained by Gram's method. The average number of bacteria in 25 phagocytes was counted and the least toxic concentration was noted where the result showed one or less than one coccus per cell
- (c) Toxicity to chick embryo —A window was cut in the shell of 10-day-old embryonated eggs,

- the shell membrane removed, and 0 3 ml of a solution of a compound (two-fold serial dilutions, using at least 3 embryos for each dilution) dropped on to the collapsed chorio-allantoic membrane. The window was sealed with a waxed coverslip and the minimum concentration causing death of the embryo within 3 to 4 days was determined.
- (d) Effect on gumea-pig skin—Intradermal injections (005 ml) were made into the shaved skin of guinea-pigs and the least concentration causing erythema or necrosis was observed. The compounds were tested also for their effect on wound healing (described later), and it was found that there was no delay in healing with concentrations up to 04 per cent (in ointment base)
- (e) Effect on rabbit conjunctiva—Applied under the eyelids of rabbits, solutions of the diamidines caused no irritation of the conjunctiva in concentrations up to 1 1,000

Antibacterial activity in vivo

By injection—Although the diamidines display high bacteriostatic and bactericidal activity against

TABLE V THE SYSTEMIC AND LOCAL TOXICITIES OF PROPAMIDINE, DIBROMOPROPAMIDINE, HEXAMIDINE, IODOHEXAMIDINE,
AND 5-AMINOACRIDINE

	Average le	ethal dose for mice	Minimum toxic concentration (g /100 ml) for			
Compound	intravenous subcutaneous		(b) Human phagocytes	(c) Chick embryo	(d) Guinea-pig skin	
Propamidine Dibromopropamidine Hexamidine Iodohexamidine 5-ammoacridine	42 10 17 6 15	55 300 62 150 100	0 6 0 1 0 2 0 1 0 01	04 08 02 04 01	0 1 0 05 0 025 0 05 0 05 0 025	

Gram-positive cocci, the ratio of antibacterial activity to systemic toxicity is such that little therapeutic activity can be demonstrated when they are administered parenterally. Hexamidine, given subcutaneously at the maximum tolerated dose, showed only slight therapeutic activity either in prolonging the survival time of mice infected with a virulent strain of staphylococci, or in reducing the incidence of kidney abscesses with a chronic strain. Against salmonella and clostridial infections in mice the results were similarly disappointing. Very slight virucidal activity was shown by hexamidine, but not by the others, against influenza virus infections in mice, but the effect was barely significant.

Locally —Compounds, in solution and in ointment base, were examined initially for local tolerance on burn-wounds in guinea-pigs by Ungar's (1944) method It was found that they did not delay the rate of healing after treatment for 4 days at concentrations twice those toxic to phagocytes Infected wounds were produced on both flanks of the shaved skin of rabbits by scarifying the skin tissue and then infecting with Staph aureus or Ps pyocyanea The wound was kept moist and enclosed in a plastic cover (Robson, 1946), solutions of the compounds were applied through a hole in the cover which could be closed with a Against staphylococcal infections the diamidines were effective in concentrations of 1 1.000, but no effect could be demonstrated at this concentration against pyocyaneal infections Better results, however, were obtained in the human subject (Kohn and Cross, 1948)

Mode of action

Bernheim (1944) found that propamidine inhibited the oxidative metabolism of bacteria, and we found that the halogenated diamidines had a similar inhibitory effect. We confirmed, also, that a 2-hour period of incubation was necessary in order to obtain maximal effects, probably in order to allow diamidines to penetrate the bacterial cell. In studying the inhibition by diamidines of oxidative systems various substrates were used, in order to discover the effect on the most important dehydrogenases present in the bacterial cell. The substrates used were glucose, sodium lactate, sodium pyruvate, sodium glutamate, sodium acetate, sodium malate, sodium succinate, and alanine

The oxygen uptake of B coli was measured in Warburg vessels after a 2-hour period of incubation at pH 78 in order to obtain optimum effects, the manometer cups contained 0 00012 M diamidine solution, 0 01 M substrate, 0 5 ml of a washed bacterial suspension of B coli containing

approximately 10^9 organisms per ml, 0.2 ml of 6 per cent potassium hydroxide, and 0.033 M phosphate buffer

It was found that the diamidines caused marked inhibition and to a similar degree with all the substrates examined. For instance, with glucose and sodium lactate as substrates the percentage inhibitions caused by propamidine after 2 hours at 37° C were 69 and 72 respectively, for dibromo propamidine the percentage inhibitions were 85 and 87, for hexamidine 83 and 80, and for iodo hexamidine 98 and 90. There did not seem to be any relationship between the degree of inhibition and the bacteriostatic activities of these diamidines, although it should be noted that only one organism (B coli) was employed in the manometric experiments

Similar experiments were carried out with tissue preparations The substrates were sodium lactate, glucose, sodium succinate, and choline chloride The lactic and glucose dehydrogenases were obtained from rat brain tissue, and the succinic and choline dehydrogenases were obtained from rat liver The manometer flasks were set up con taining 1 ml of 0 022 M phosphate buffer pH 78, 02 ml of 0.01 M substrate, 0 00012 M (and 0 0012 M) diamidine solution, Locke's solution, 1 ml of enzyme preparation, and 02 ml of 6 per cent potassium hydroxide, the volume was adjusted to 3 ml The flasks were incubated at 37° C for two hours No inhibition of oxygen uptake was observed with any of the substrates except choline, with which 72 per cent inhibition was obtained

In bacteriostatic experiments, with staphylococci in blood, p-aminobenzoic acid had no inhibitory effect on the bacteriostatic activity of propamidine, hexamidine, or their halogenated derivatives But Bichowsky (1944) found that nucleic acid reduced the antibacterial action of propamidine, and Elson (1945) suggested that phospholipides competed with diamidines for the anionic position on the We found in manometric experiments with sodium lactate as substrate that 0.1 per cent of nucleic acid reduced the inhibitory effect of prop amidine on the oxygen uptake of B coli by 14 per cent after 1 hour at 37° C and by 43 per cent after 2 hours It is suggested, therefore, that the diamidines may act by deranging some phase in the metabolism of bacteria involving nucleic acid

DISCUSSION

The diamidines are interesting compounds since they have such a wide range of action—they are effective not only against protozoa but also against bacteria and fungi. The use of the diamidines as local bactericidal compounds requires the fulfilment of certain conditions—ie, the compounds should show high bacteriostatic and bactericidal activity, they should be effective in body tissues, and they should be well tolerated locally Tests should parallel as far as possible the conditions of actual use, results obtained, therefore, in serum or blood have more significance than those in broth, and in surface infections local toxicity to tissues is of more importance than systemic toxi-Although the diamidines are regarded as potentially toxic compounds for parenteral use they are well tolerated when applied locally, the small amounts absorbed from the surface are unlikely to produce any systemic effects

In the investigation of many substituted diamidines it was found that the introduction of halogen in the 2-position into one or both benzene nuclei had a favourable effect. The introduction of two halogen atoms into the same benzene nucleus in the 2 6 positions was less beneficial the minimal effective concentration for 2-10dopropamidine against staphylococci in blood was 4 ug/ml, whereas the minimal effective concentration for the 2 6 diiodo derivative was 16 μ g /ml The halogenated stilbene compounds possessed less bacteriostatic activity than the diphenoxyalkane compounds, the halogenated diphenyl ethers were even less active (Table I) We observed that the homologous diguanidines displayed the same gradation in bacteriostatic activity as the diamidines, similar results have been obtained by Fuller (1942)for aliphatic amidines guanidines

In an attempt to elucidate their mode of action we found that the diamidines exerted a general inhibitory effect, to a similar degree, on a fairly wide range of oxidizable substrates. The number of substrates involved may be an indication that an effect was exerted directly on a respiratory mediator common to a number of systems, and not on a specific dehydrogenase enzyme system parallelism was found between the effects obtained with tissue and bacterial enzymes, there may, however, be some relationship between the effects of the diamidines in causing both an inhibition of choline oxidase and fatty degeneration of the liver The experiments with induced drug-resistant strains of bacteria threw little light on the problem except to emphasize that dissimilar substances like penicillin and the diamidines probably have different types of action, since an organism made resistant to one diamidine was also resistant to other diamidines but was sensitive to penicillin

Gale (1947) has demonstrated a relationship between the assimilation of glutamic acid by staphylococci and their sensitivity to penicillin, but we have not as yet investigated whether diamidines can similarly block glutamic acid assimilation

SUMMARY

- 1 A study of the antibacterial properties of the diamidines showed that bacteriostatic activity in the diphenoxyalkanes rose to a maximum from the propane to the hexane and nonane derivatives. This increased bacteriostatic activity was accompanied by an increase in intravenous toxicity, but by only a relatively small increase in local toxicity to phagocytes. Gram-positive bacteria were more susceptible than Gram-negative bacteria, and the bacteriostatic activity was maintained in the presence of blood.
- 2 The introduction of halogen into one or both benzene nuclei in the diphenoxyalkanes further increased the bacteriostatic effect against Staph aureus as well as against B coli, Proteus vulgaris, and Ps pyocyanea, with little alteration in local toxicity
- 3 Two new derivatives, dibromopropamidine and iodohexamidine, were studied more closely, in comparison with their parent compounds, for their possible use in surface infections. They showed both bacteriostatic and bactericidal effects which appear to be due to inhibition of the oxidative metabolism of bacteria. Small differences in pH markedly influenced both the inhibitory and the bacteriostatic effects, which were increased in an alkaline medium and decreased in an acid medium.
- 4 Drug-resistant strains of bacteria could readily be induced by repeated subcultivation in vitro, a diamidine-resistant strain, although resistant to other diamidines, was sensitive to penicillin and a penicillin-resistant strain was sensitive to the diamidines

We are indebted to Miss Pattinson, Mrs Bradish, and Mrs Oakley for valuable assistance, to Dr Gordon and Miss Sowden for the biochemical part of this investigation, to Dr J N Ashley and his colleagues for the preparation of the compounds, to Dr W R Thrower for initiating the clinical trials, and Dr A J Ewins for his interest throughout the investigation

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Since the completion of this paper we have noted an article by Bichowsky-Slomnitzki (J. Bact., 1948, 55, 33), who found that nucleic acid and certain polyamines (spermine and spermidine) antagonized the inhibitory effect of diamidines (stilbamidine and pentamidine) on the growth of B coli It was assumed that the diamidines caused metabolic disturbances of the cell nucleotides by fixation of nuclear substances, whereas a different mechanism of action existed in the antagonism between diamidines and polyamines, which was probably asso ciated with their competition for the same cellular substance

THE ACTION OF THE VENOM OF A MEXICAN SCORPION (CENTRUROIDES NOXIUS, HOFFMANN) ON CHOLINESTERASES

BY

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From the Physiological Institute, Cambridge

(Received December 4 1947)

Some of the effects of scorpion venom resemble those of eserine and other cholinesterase inhibiting substances For instance the venom augments the tension response of skeletal muscle to maximal motor nerve volleys and may produce spontaneous contractions (del Pozo and Anguiano, 1947) potentiation was shown to be due to repetitive response of the muscle fibres similar to that produced by eserine (Burns and del Pozo, 1947) an anticholinesterase property of the venom were the cause of this effect, the venom should also produce eserine-like actions on other organs For instance, substances which act by inhibition of cholinesterase produce in the isolated intestine a characteristic contraction which develops slowly and disappears only gradually when the drug is washed out The effect contrasts with the immediate and quick contractions produced by drugs acting directly on the smooth muscle The effect of venom was therefore examined on the isolated intestine of the guinea-pig and rabbit In addition, in vitro experiments were performed to find out if the activity of true- as well as pseudo-cholinesterase could be affected by the venom

METHODS

Pieces of the guinea-pig's and rabbit's small intestine were suspended in 16 cc Mg-free Tyrode solution. The contractions of the fibres of the longitudinal muscle layer were recorded by a Lovatt Evans frontal writing lever. The contractions of the circular muscle layer were observed with the naked eye through the glass wall of the tank, and were seen to cause a lengthening of the preparation. Thus relaxation of the longitudinal muscle layer and contractions of the circular layer both affected the record in the same direction. The bath was emptied by overflow and the substances were added in 0.2-0.5 cc saline, with a syringe. Air was bubbled continuously through the Tyrode solution, the temperature of which was kept between 32 and 36° C

Human serum was used as a source of pseudocholinesterase and minced tissue of the caudate nucleus of rabbits as a source of true-cholinesterase Since human serum contains practically no true- and the tissue of the caudate nucleus practically no pseudo-cholinesterase, their effects on acetylcholine hydrolysis are due in each case to one of the two enzymes only The effect of venom was compared with that of eserine, which inhibits both cholinesterases (Hawkins and Mendel, 1947)

The procedure adopted was as follows liminary experiments the amounts of cholinesterase preparation necessary to hydrolyse in 10 min about 90 per cent of 200 or 400 μ g of acetylcholine at 37° C Acetylcholine was estimated with were determined the eserinized frog rectus muscle It was found that 02 to 025 cc human serum or the equivalent of about 10 mg tissue of the caudate nucleus made up in a final volume of 3 to 35 cc were required for this purpose Several samples were then set up Each contained the same amount of simultaneously the cholinesterase preparation, to which was added either venom or eserine in varying concentrations, the total volume was then made up to 2 or 25 cc with saline Ten minutes later the acetylcholine (in 1 cc) was added to the samples, these were kept for another 10 min before the enzyme action was stopped by acidification and boiling. The samples set up with true cholinesterase (a suspension of finely ground brain tissue) were shaken during the whole 20 min The acetylcholine content of the samples after neutralization was assayed on the eserinized frog rectus muscle The venom present in some of these samples was found not to interfere with the assay

The scorpion venom was obtained from ground telsons of a Mexican scorpion (Centruroides norius Hoffmann) by extraction with saline and precipitation with acetone (Anguiano, 1947) A yellowish greenish powder is obtained which is stable and soluble in saline. The certainly lethal dose of the powder on intravenous injections into 20 g mice was 11 mg, this dose killed the mice within 15 to 30 min

RESULTS

Isolated intestine preparations

Gunea-pig's intestine—The addition of 2 to 10 mg of venom extract to the 16 cc bath caused,

after a latent period of 30 to 60 sec. a slowly developing contraction of the longitudinal muscle After washing out the venom, relaxation proceeded gradually (Fig 1b) The effect resembled that of eserine by its long latency, its gradual development and disappearance, it differed. however, from that of eserine in the following respects After replacing the bath with fresh Tyrode solution the effect of eserine subsided even more gradually that that of the venom extract (see Fig 1a and b) In addition eserine has a more powerful action on the circular muscle The preparation, first shortened by eserine owing to the contraction of the longitudinal muscle, frequently shows short periods of lengthening as a result of strong contractions of the circular muscle layer This is seen in Fig 1a during the first four minutes of the eserine action. With the scorpion venom. contractions of the circular muscle may be absent or occur only once or twice In the record of Fig 1b one such period of lengthening owing to contraction of the circular muscle is seen after the administration of the venom

Approximately 5 mg of venom were found to have an action equal to or somewhat smaller than that of $10~\mu g$ of eserine, i.e., eserine was about 500 times more active than the venom extract. On repeated administration the venom did not lose its power to cause contraction of the smooth muscle in the intestine, thus no desensitization to the

venom took place, unlike the characteristic desensitization which occurs with snake venoms

Atropine had an antagonistic effect on the action of the venom extract, but the antagonism was less pronounced than with eserine. In the experiment of Fig. 1, atropine 1 in 15 million had abolished the action of 5 and even of 10 mg of venom extract, but 20 mg of venom extract still produced a pronounced contraction, on the other hand the preparation had become practically insensitive to $150~\mu g$ of eserine (at c). In some experiments one part of atropine in a million reduced but did not abolish the effect of even 5 mg of venom extract

Rabbit's intestine — The effect of the venom extract on the rabbit's intestine resembled that on the guinea-pig's intestine The addition to the 16 cc bath of 1 mg extract and, in some preparations, even of 0.5 mg produced small contractions Stronger effects were obtained with the addition of 5 to 10 mg of venom extracts When these amounts were kept in the bath for a few minutes a gradually developing contraction of the longi tudinal muscle layer with usually slight contractions only of the circular muscle occurred after a latent period of 30-90 sec Relaxation of the muscle after washing out the extract proceeded as gradually as after eserine, the venom also produced a contraction of the circular muscle layer shortly after being washed out, but to a less extent than eserine In nearly all experiments the effect

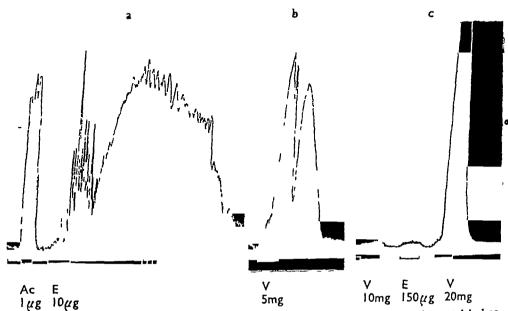


Fig 1—Contractions of the isolated guinea-pig's intestine in 16 c c bath Ac, acetylcholine washed out after 1 min V and E scorpion venom extract and eserine sulphate respectively washed out after 2 min a and b before, c during atropine sulphate 1 in 15 million Time in 30 sec indicated in a. For details see text)

SCORPION VENOM ON CHOLINESTERASE

on the rabbit's intestine of 5 mg venom extract was slightly weaker, and that of 10 mg much

stronger, than that of 10 µg of eserine Atropine had only a slight antagonistic action on the effect of venom It was even less effective in antagonizing the action of the venom extract than in the corresponding experiments on the guineapig's intestine The effect of 5 mg venom extract was only slightly if at all reduced by a concentration of atropine (1 10°) which abolished the effect of 10 µg eserine sulphate

Cholinesterase preparations

Venom extract had only a slight inhibiting action on pseudo- as well as on true-cholinesterase the experiments of Table I a 01 per cent solution of the venom had no effect on the enzyme activity in the samples and 1 to 2 per cent solutions caused partial inhibition When this effect was compared with the inhibition produced by eserine sulphate it was found that, at room temperature, eserine 1 in 80 millions had a much weaker and eserine 1 in 20 millions a slightly stronger action than 1 to 2 per cent venom extract, in the one experiment with true-cholinesterase carried out at 37° C the difference in potency between the two substances was even greater

The extract of venom of the Mexican scorpion used in the present experiments exerted an anticholinesterase activity only if tested in very high concentrations, and there was no difference between its effects on pseudo- or true-cholinesterase When the inhibitory action of the venom extract on these cholinesterases was compared with that of eserine in in vitro experiments preparations

it was found that, weight for weight, the venom extract was 200,000 to 800,000 times less potent The necessity of using high concentrations of venom extract for demonstrating its cholinesterase inhibitory action raises the question of how far the effect is due to the venom proper or to impurities present in the extract or, on the other hand, how far such impurities may have reduced any inhibitory action of the venom proper This question can be answered only if purified preparations of the venom become available

It is difficult to see how the feeble cholinesterase inhibiting action of the venom extract can contribute more than to a slight extent to the smooth muscle contracting effects on the isolated intestine The effect has a superficial resemblance to that of eserine, but 0.5 mg of the venom extract were found to be about as potent as 1 µg of eserine sulphate, whereas in the in vitro experiments on cholinesterase preparations 1 µg of eserine was as potent as 200 to 800 mg of venom extract addition the smooth muscle contracting effect of the venom was more resistant to atropine than that The action of the venom extract on the intestinal preparations appears therefore to be independent of its feeble cholinesterase inhibiting Similarly the spontaneous contractions of skeletal muscle, and the potentiating effect which the venom extract has previously been found to exert on the twitch response to single motor nerve property volleys in cats, cannot be attributed, or can be attributed to a slight extent only, to inhibition of These "eserine like" effects of the venom extract can more reasonably be explained as an action either directly on the motor nerve endings or on the motor end plates or on both cholinesterase structures

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In experiments (1) to (3) 200 µg acetylcholine were used as substrate, in (4) 400 µg

SUMMARY

The effects of extracts of the venom of a Mexican scorpion (Centruroides noxius, Hoffmann) were examined on cholinesterase preparations with the view of finding out if the "eserine-like" effects this venom exerts on isolated tissues and in animals on intravenous injections could be explained by a cholinesterase inhibiting property. The venom extract was only found to inhibit pseudo- as well as true-cholinesterase when present in very high concentrations. It is concluded that the symptomatology of the venom poisoning is independent of, or dependent to a very slight degree only on, inhibition of cholinesterase.

I should like to make grateful acknowledgment to Dr W Feldberg for his help and interest in this work, to Prof E D Adrian for the hospitality afforded in his Institute, and to the British Council for their invitation to come to England

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THE ACUTE DISTRIBUTION OF INTRAVENOUSLY ADMINISTERED LEAD ACETATE IN NORMAL AND BAL-TREATED RABBITS

BY

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(Received December 24, 1947)

Little information is available about the actions of 2 3-dimercaptopropanol (BAL) in poisoning by lead salts, and so far as is known nothing has been published about its effect on the distribution of lead in the body. This paper provides some new observations on the distribution of lead, indicated by the use of Pb²¹² (thorium B) as a tracer, and on changes in the distribution after the administration of BAL

METHODS

Radioactive lead was obtained from the deposit on a charged button exposed to a thorium source A button with Pb212 (intensity of about 1 millicurie) was heated gently in a slightly acidified 0 01 per cent (w/v) solution of lead acetate, so that the Pb212 deposit was exchanged for part of the lead in the solution For administration to rabbits a solution was prepared such that the volume injected (2 0 ml) contained 2 07 mg of lead (as acetate) per kg body weight (i e 001 mM/kg) incorporating 100-300 microcuries of Pb212 dissolved in 4 per cent (w/v) dextrose Young rabbits of both sexes and various breeds and of average weight 1 3 kg were used. Injections were made into the marginal vein of one ear After injection the rabbits were placed in metabolism cages and allowed access to water but not food Their bladders were emptied by suprapubic pressure before injection and six, twelve, and eighteen hours afterwards, unless the rabbits were killed earlier The animals were killed by a blow on the head one, six, or twenty-four hours after the injection of lead acetate Solutions of BAL were freshly prepared in 66 per cent (v/v) aqueous propylene glycol and were injected into the paravertebral muscles as discussed below

Immediately after death the thorax-was opened, and blood was collected by bleeding from the great veins Clotting was prevented with heparin, and the samples were centrifuged immediately. The organs were then dissected, starting with those in which a low content of

lead was expected, and taking care to avoid contamination between different tissues Complete organs were washed with water, dried of superficial moisture between filter papers, and weighed, they were then chopped finely and, in organs weighing less than 10 g, ashed entire From other tissues samples weighing 2 to 10 g were ashed Liver samples were taken from well-mixed choppings of the entire organ Muscle samples were taken from the outer part of the thigh Bone epiphysis and diaphysis were obtained from the long bones of one hind limb and one fore limb Marrow was completely removed from the central cavities of these bones and estimated separately The samples of diaphysis were therefore practically marrow-free, the values for epiphysis, ribs, vertebrae, and skull vault were influenced by the amount of marrow contained in the cancellous tissue The injected ear and the small piece of cottonwool used to control bleeding were taken as an independent sample, and in the majority of experiments the uninjected ear was taken as a control. The amount of lead lost by leakage at the site of injection could therefore be assessed, the average amount was slightly more than 2 per cent of the dose Duplicate samples were taken from the liver and one other organ, from these duplicates an estimate of the error of the lead determinations was

After being weighed the tissues were placed in longnecked flasks containing 40 ml concentrated nitric acid and 20 ml 60 per cent (w/w) perchloric acid, and allowed to stand for at least 30 minutes. The flasks were then heated cautiously until the contents were boiling, care being taken to prevent the formation of excessive amounts of foam. The tissues dissolved and the solutions were boiled gently until about 20 ml remained, when a further 30 ml of concentrated nitric acid was added. Boiling was continued until about 10 ml of clear solution remained. In all, the solutions were boiled for 1½ to 3 hours. If charring occurred during this process the flasks were allowed to cool and a few drops of fuming nitric acid or hydrogen peroxide were added until a clear solution was obtained

The contents of the flasks were washed into beakers and the volume made up to about 100 ml with water The reaction of the solution was adjusted to neutrality with 40 per cent (w/v) sodium hydroxide (using B D H Universal Indicator), 0.25 ml of concentrated hydrochloric acid was then added, followed by 0.5 ml of 1 12 per cent (w/v) lead acetate The lead was then precipitated as lead sulphide by the addition of 10 ml of 10 per cent (w/v) sodium sulphide In order to avoid the precipitation of calcium phosphate from asked bone samples on the addition of the 40 per cent sodium hydroxide, a slightly different procedure was adopted the alkalı was added until the phosphate began to be precipitated and then just enough concentrated hydrochloric acid to redissolve it was added with vigorous stirring, lead acetate and sodium sulphide were then added as described above

The precipitates of lead sulphide were allowed to stand for at least one hour before filtering under moderate suction in small Hirsch funnels. The precipitates were collected on small circles of filter paper 2 cm in diameter. They were washed once with distilled water and once with acetone to carry down the portion adhering to the sides of the funnel and to facilitate drying. The papers were removed when dry and the funnels were washed, first with a small quantity of concentrated hydrochloric acid and then with distilled water. The filtrates and funnel washings were then neutralized, hydrochloric acid, lead acetate, and sodium sulphide were added, and the fresh precipitates were filtered as described above.

The radioactivity of the precipitates so obtained on paper circles 2 cm in diameter was estimated by means of a Geiger-Muller counter of the usual bell-shaped type with a thin, supported mica window, a paper screen being used to absorb the α particles. The counter was calibrated at each experiment with samples prepared from aliquots of the solution used for injection by precipitation with a lead sulphide carrier as described above. In preliminary experiments it was found that the total count per minute, corrected for the background, was proportional to the amount of radioactive solution used, over the wide range employed in the biological experiments, and that appreciable changes were not produced by doubling or halving the amount of lead carrier used

From the counts obtained on aliquots of the injected solution and the counts on individual samples under identical conditions, the proportion of the dose per gramme of tissue, and hence the lead concentration per gramme of tissue, were calculated. The total amounts of lead in organs of known weight were calculated from the data, and approximate values were obtained for other tissues on the assumptions that the total blood volume was 70 ml/kg, the total weight of bone marrow 20 g/kg (Nye, 1931), the total weight of bone without marrow 60 g/kg, the total weight of skin 120 g/kg, and the total weight of skeletal muscle 520 g/kg (Levine, Mann, Hodge, Ariel, and Du Pont, 1941) With one exception the lead concentration in bone was

taken as the value obtained from diaphysis, as being the sample freest from marrow

By taking two precipitates the error of the estimates was considerably reduced, since a small proportion of the lead sulphide of the first precipitate, lost either because it slipped under the paper or because the particles were too fine to be retained, was recovered in the second precipitate. Also the ratio between the Geiger-Muller counts for the first and second precipitates (generally about 10) gave an indication of the reliability of a particular estimate. The standard error of the lead estimations, calculated from duplicate determinations made during these experiments, was ± 3 per cent. The mean recovery of lead from the entire animal, calculated as described above, was 90 per cent with a standard deviation of ± 10 per cent of the dose

RESULTS

Data are presented for the distribution of lead in fourteen rabbits, all of which received a single dose of lead acetate containing 2 07 mg of lead per kg of body weight and eight of which were treated with BAL Of the rabbits which did not receive BAL, two were killed at one hour after injection, one at six hours, and three at twenty-four hours. Of the treated rabbits, two were given 50 mg/kg BAL (1 e 40 molecules per atom of lead) immediately after the lead acetate and were killed at one hour One rabbit was given 50 mg/kg one hour after the lead acetate and 125 mg/kg four hours later and was killed at six hours. Two were treated in the same way but were not killed until twenty-four Three more were treated with BAL 50 mg/kg nineteen hours after the lead acetate and 12.5 mg/kg at twenty-three hours and were killed at twenty-four hours The doses of BAL used were very near the toxic range, as maximal effects were being sought one rabbit of the last group (No 93) died nearly twenty-three hours after receiving lead acetate, shortly before the small injection of BAL was due Ill effects were not observed in any of the other rabbits

The concentrations of lead in microgrammes per gram ne of tissue and the percentages of the dose found in various organs and calculated for various tissues are shown in Tables I and II Considerable variation occurred between different rabbits which received the same treatment, especially at twenty-four hours, but from the general trend of results it appeared that with a few exceptions the distribution of lead when BAL was not given did not alter greatly between one and twenty-four hours. The exceptions were the plasma and lungs in which the lead content showed a steady decline, and in the contents of the alimentary canal, in which the amount of lead increased in the longer experiments Changes in other organs did not exceed the variation

THE FFECT OF TREATMENT WITH BAL ON THE CONCENTRATION OF LEAD IN THE TISSUES OF RABBITS ONE HOUR, SIX HOURS, AND TWENTY-FOUR HOURS AFTER THE INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2 07 MG Pb/KG) TABLE I

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μg lead per g	hours	50 mg/kg at 1 hr 12.5 mg/kg at 5 hr	82 ç 1 05	0 18 37 6 137 6 137 7 273 8 17 0 035 10 5 3 00 3 10 4 2 7 4 2 7 6 0 0 7 7 0 0 7
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	,	ž	74 & 1 20	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
Time after	giving lead	Treatment with BAL (intramus- cular injection)	Rabbit No Weight, kg	Plasma Erythrocytes Spleen Bone marrow Liver Bile Stomach contents Small intestine contents Colon contents Kidneys Lungs Heart Skeltal muscle Diaphyses Ribs Vertebrae Skull vault Brain Skin

· Pregnant

‡ The injected solution contained some particulate matter presumably basic lead acetate

[†] Died 22 hr 40 min after injection of lead acetate

TABLE II

THE EFFECT OF TREATMENT WITH BAL ON THE DISTRIBUTION OF LEAD IN RABBITS ONE HOUR, SIX HOURS, AND TWENTY-FOUR HOURS AFTER THE INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2 07 MG P_b/RG)

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		mg /kg 1 5 mg /kg 2 after leac	94∥ ♀ 1 25	0 0 36 0 36 0 36 0 36 0 36 0 36 0 05 0 05	94%
		50 12 5	93§ ¢ 1.25	\$\\ \begin{align*} \ \ 1 & 0 & 66 \\ 13.0 & 0.31 \\ 1.26 & 0.28 \\ 0.0074 \\	%58
	nrs	kg 1 hr /kg 5 hr ·lead	95 p 1 50	$ \begin{array}{c} $	102%
ale	24 hours	50 mg /kg 1 h 12 5 mg /kg 5 h after lead	92 ở 1 15	0007 0377 0037 0037 0072 0072 0073 0074 0074 0074 0074 0074 0074 0074	74%
an or tiss			91 ¢ 0 98	2 42 2 42 7 08 519 0.29 0.29 0.26 0.26 0.26 0.26 0.26 0.26 0.26 0.26 0.26 0.26 0.26 0.27 0	82%
ntire orga		None	90 s 1 00	000 0167 0167 0167 0005 0005 0010 0010 0021 0022 0022 0022	93%
dose in e			86 9 1 40	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	%11
Percentage of dose in entire organ or tissue	urs	50 mg/kg at 1 hr 12 5 mg/kg at 5 hr	82 g 1 05	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	104%
Perc	6 hours	None	83 ♀ 1 10	\$\\ \frac{001}{2555} \\ \frac{011}{142} \\ \frac{002}{002} \\ 00	%86
		kg at er lead	79 ¢ 1 22	2 54 2 54 0 005 7 63 33 0 0 006 0 36 0 30 0 002 0 135 10 3 2 32 0 005 0 14 0 14 0 14 0 14	%06
	ūr	50 mg /kg at once after lead	75 g 1 15	4 48 1 48 0 26 8 12 40 1 0 85 - 6 40 0 0 10 6 60 0 10 6 60 0 10 1 83 0 100 0 12 0 100 0 12 0 100 0 12	%91
	1 hour	ne	76 9* 1 70	145 277 277 168 568 0007 010 037 0006 052 062 062 062 163 163 143 135 143 1000e	85%
	and a second	None	74 ở 1 20	125 169 260 677 0006 0099 011 150 113 0018 125 744 0018 0018	103%
Time after	giving lead	Treatment with BAL (intramus- cular injection)	Rabbit No Weight, kg	ts s	l'otal recovery

Pregnant
 Based on concentration in diaphyses Excluding marrow
 Based on half concentration in epiphyses Excluding marrow

§ Died 22 hr 40 min after injection of lead acetate || The injected solution contained some particulate matter presumably basic lead acetate between different rabbits killed at the same time 50-70 per cent of the entire dose was found in the liver, where, as in the bone marrow and the spleen, the highest concentrations of lead $(10-50~\mu g/g)$ occurred. Concentrations of $1-5~\mu g/g$ occurred in the red cells, lungs, kidneys, and bone. All the other tissues regularly sampled contained about 0.05 to $0.5~\mu g$ of lead per gramme. A small amount of lead was excreted in the urine (Fig. 1), less than 1 per cent in twenty-four hours and to judge from the movement of lead in the gut contents, another 1 per cent or so was likely to be excreted in the faeces on the second and third days

When BAL was administered several differences in the distribution of lead were observed content and concentration of lead in the liver, spleen, and bone marrow were consistently reduced, except in the liver at six hours Data at six hours have been obtained for only one normal and one BAL-treated rabbit, the closely similar concentrations found in the livers do not seem to be an outstanding exception to the thesis that BAL reduces the amount of lead in the liver The concentration of lead in red cells was also consistently lower after BAL, but this effect was offset by an increase in the amount in the plasma, so that the effect on total blood lead was variable On the other hand, the excretion of lead in the urine was increased five- or tenfold (Fig. 1 and Table III) The increase was still apparent between twelve and twenty-four hours even when BAL had not been given after the fifth hour In view of the usually transient effects of BAL on the excretion of other metals (Wexler, Eagle, Tatum, Magnuson, and

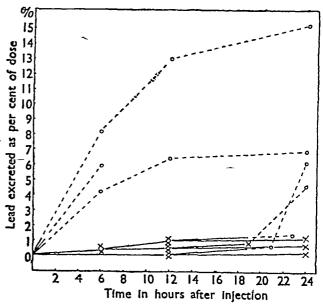


Fig 1—The effect of BAL on the cumulative excretion of lead in the urine of rabbits after the intravenous administration of lead acetate (2 07 mg Pb/kg)

Ordinates amount of lead excreted as a percentage of the dose administered (1% = 20.7 μ g Pb/kg body weight) Abscissae time in hours after injection of lead BAL given intramuscularly, either 50 mg/kg at 1 hour and 12.5 mg/kg at 5 hours, or 50 mg/kg at 19 hours and 12.5 mg/kg at 23 hours

Watson, 1946, Eagle, Magnuson, and Fleischman, 1946, Eagle, Germuth, Magnuson, and Fleischman, 1947) this finding was somewhat unexpected In the rabbit which died at twenty-three hours (No 93)

TABLE III THE URINARY EXCRETION OF LEAD AFTER INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2 07 Mg $\,$ Pb/kg) to rabbits, with and without subsequent intramuscular injections of Bal

Rabbit No	50 mg/kg at (hrs	12 5 mg /kg at (hrs	Urine volume ml at hours after injection of lead			Urine		entration μ rs after of lead	g./ml	
	after lead)	after lead)	0-6	6–12	12–18	18-24	0-6	6–12	12-18	18-24
83 86 90 91 93 94 97 82 92 95	nil nil nil nil 19 19 19	nil nil nil nil 23 23 5 5	50 50 0 18 24 22 20 12 10 20	5 28 	8 30† 12 	50* 10‡ 18	0 06 0 15 0 36 0 39 0 31 10 7 10 0 12 8	0 14 0 04 0 91 0 3 0 20 10 7 6 4	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	0.29* 0.29* 4 35‡ 5 77

The heavy black lines in the body of the table separate values before injection of BAL from values after its injection

^{* 18} hours-22 hours 40 minutes after injection of lead acetate † 12-21 hours after injection of lead acetate

^{‡ 21-24} hours after injection of lead acetate

the one dose of BAL failed to increase the excretion of lead in the urine Excretion into the alimentary canal was also greatly increased, but the data are insufficient to show whether the increase was primarily due to biliary or to intestinal excretion Precautions were not taken to prevent coprophagy, and it is possible that some or all of the lead in the stomach contents got there in that way Increased concentrations of lead were regularly found in the skeletal and cardiac muscle and in the stomach wall the wall of the intestine increased amounts of lead were found in animals killed shortly after administration of BAL, but in the rabbits (Nos 92 and 95) to which BAL was given at one and five hours and which were killed at twenty-four, any increase produced by BAL had disappeared In other tissues changes were small or variable. For example, bone diaphysis and lungs showed a decrease in lead content at one hour and an increase at twenty-four In rabbit No 94 the injected solution was observed to be cloudy, and it appears likely that the exceptionally high concentration in the lungs was due to retention of some particulate matter, probably basic lead acetate, rather than to the action of BAL

The various changes have been summarized in Table IV, in which tissues having common physiological functions and similar concentrations of lead, and in which the lead concentrations were similarly affected by BAL, have been grouped together. The oversimplifications involved are perhaps justified by the more immediate comprehensibility of the data so presented.

TABLE IV

Su mmary table of the distribution of lead after intravenous administration of lead acetate (2 07 mg Pb/kg) to rabbits with and without subsequent intramuscular injections of BAL. The figures given are the mean percentages of the dose found in each group of tissues for all similarly treated rabbits. Details of treatment are as shown in Tables I, II, and III

Distribution at	1 hour		6 hours		24 hours		
Treatment No of Rabbits	No BAL 2	BAL 2	No BAL 1	BAL 1	No BAL	Early BAL 2	
Liver, spleen, and bone marrow Alimentary canal and contents,	80	45	76	75	68	52	54
faeces, and bile Urine Blood Skeletal muscle Bone	0 5 -4 1 5 6	14 - 7 8 2	0 7 0 1 3 1 12	4 5 6 1 6 8	3 0 7 2 1 7	7 11 1 3 9	8 3 1 5 10

DISCUSSION

The extensive literature on the distribution of lead in animals contains few observations on the immediate fate of a single dose of a soluble inorganic salt administered intravenously. The most comprehensive data appear to be those of Weyrauch (1931) and of Kehoe and Thamann (1933) for rabbits and of Behrens (1925) for mice and cats. In general the present results are consistent with those already published (Table V) for the rabbit, at least in order

TABLE V

Distribution of lead one hour after intravascular injection of lead salts (after various authors) The figures given have been deduced from the published data in accordance with assumptions used elsewhere in this paper Kehoe and Thamann's data are taken from rabbits killed half or two hours after the injection of lead

		Per cent of dose in various tiss					
Author	Ве	hrens (1	925)	Kehoe and Thamann (1933)	Ginsburg and Weatherall (1948)		
Species Lead salt	Mouse Chlor ide	Cat Chlor ide	Cat Chlor- ide	Rabbit Chloride	Rabbit Acetate		
dose (mg Pb/kg)	15	4	0 04	7–17	2 \		
Blood Spleen	20	- 35	25 0 2	2 5 2 5	3 5 1 5		
Liver	29	18	20	85	$6\hat{2}$		
Intestine	īi	3	-ĕ	5	0 5		
Kidneys	9	15	4	15	1		
Lungs			0.6		0 9		
Heart	07	2*	0 4*		0 03		
Muscle	10	20	5	85	1 5 6		
Bone Brain		_]	20	6 5	0 02		

*Lead given by intracardiac injection

of-magnitude However, certain discrepancies are larger than might be expected from differences in dosage and in experimental technique Except for a single figure of Behrens's for cats (loc cit, p 353), bone and marrow apparently have been taken together by previous authors, in unspecified proportion and usually from unspecified sites experiments reported here, bone marrow has been found to contain up to eight times the amount or twenty times the concentration of lead in bone, particularly when relatively marrow-free diaphyses were sampled It follows that estimates for bone and marrow taken together mean little, as small changes in the proportions of the two tissues in a sample must greatly influence the result Differences in the lead content of different bones and different

parts of the same bone in acute experiments are partly explicable on this basis Behrens's one figure for the lead concentration in the bone marrow of a cat is considerably lower than that given for bone This finding is the opposite of in the same animal all the present observations, and apart from the obvious possibility of a species difference no explana-The present findings are also tion is apparent directly the opposite of those described in chronic poisoning in cats, and perhaps rabbits, by Aub, Fairhall, Minot, and Reznikoff (1925) In other tissues the present values for blood and for the alimentary canal are low when compared with the general trend of results Weyrauch's data are given suitably amended they for dry weights of tissue appear quite consistent with those here Kehoe and Thamann's recoveries in their five experiments with lead chloride lasting less than twenty-four hours average 44.5 per cent, and while they have admittedly discarded their "remainder" it is difficult to account for up to (in one case) 88 per cent of the dose outside the liver, spleen, kidneys, washed intestinal tract, blood, muscle, bone, and central nervous system It seems more probable that losses occurred during administration or estimation, and consequently that their values err towards the low side Some figures of Brady (quoted by Aub et al, 1925) for the lead content of the livers of anaesthetized rabbits with cannulated bile ducts after considerably larger doses of lead show, like the present data, about 50 per cent or more uptake of lead by that organ in the first few hours after intravenous administration Behrens's figures indicate that in mice the uptake and excretion of lead by the kidney is much more rapid than in rabbits, and that excretion through the alimentary canal is also much greater—the concentration per unit dose remaining in the tissues is correspondingly Urinary excretion in the present experiments has been fairly steady, amounting to not more than 1 per cent of the total dose in twenty-four hours The early disappearance of lead from the plasma agrees with the observations of Bambach, Kehoe, and Logan (1942), and with those of Mortensen and Kellogg (1944) in dogs and guinea-pigs

It should be noted that the present work was done with young rabbits Kasahara and Arimichi (1934) found higher concentrations of lead in the blood of rabbits weighing about 10 kg than in rabbits of double this weight fed on the same relative amounts of lead. Their data can be interpreted as due to differences either in the rate of absorption or of deposition and excretion, but they indicate some difference in the metabolism of lead by young and old animals. It should also be noted

that the amount of radioactive material used in the present experiments was large. The possibility exists that some injury was done to those tissues in which the material was most highly concentrated, and that the affinity of the tissues for lead was consequently altered, but it seems unlikely that such an effect would be produced in the short duration of these experiments

There is at present very little indication how BAL produces the described changes in the distribution Some of the changes are probably due to the actions of BAL on the rabbit In view of the cardiovascular effects of BAL (Chenoweth, 1946), it is not surprising to find an increased amount of lead in the splanchnic area after simultaneously administered BAL But such actions are likely to be less important when lead has been fixed by the tissues, as appears largely to have happened within an hour Changes observed after this time more probably depend on the relative dissociability of the compounds formed between lead and the tissues and between lead and BAL, on the behaviour of the undissociated lead-BAL compound or compounds, and on the amount of BAL present in a The importance or otherwise of the given tissue last factor is illustrated by considering the data of Peters, Spray, Stocken, Collie, Grace, and Wheatley (1947), who showed that in a rat one hour after a dose of 100 mg/kg of BAL containing S35, 28 per cent of the dose was found in the liver and 0 17 per cent in the brain If a similar distribution of BAL is assumed in rabbits poisoned with lead, the amount of BAL in the liver of the rabbits described here would not have greatly exceeded one molecule per atom of lead, even if all the S³⁵ were present as unaltered BAL, whereas the amount of BAL in the brain would have been a two-hundredfold excess In spite of this, BAL had a greater effect on the lead content of the liver than on that of the brain Without data about the distribution of BAL in the conditions of the present experiments, detailed consideration on these lines is not profitable

Weatherall (1948) has shown that, after a single dose of lead acetate by stomach tube in rabbits, BAL diminishes the subsequent anaemia, increases the coproporphyrin excretion, and does not greatly affect the mortality. The present observations are consistent with Weatherall's hypothesis that BAL acts by preventing the uptake of lead ions by red cells, and they indicate moreover that BAL can probably remove lead from cells which have already taken it up. On the other hand, no light is thrown on the increased coproporphyrin excretion, nor on the increased mortality produced by BAL in subacute lead poisoned rabbits (Braun, Lusky, and

Calvery, 1946), nor on the prevention of the renal effects of lead in rats (Chiodi and Sammartino, 1947) Further and more chronic experiments are necessary to provide evidence on these points

SUMMARY

1 The distribution of lead in the tissues of rabbits after intravenous injection of lead acetate (2 07 mg Pb/kg) has been studied by the use of the short-lived isotope Pb¹¹² (thorium B)

2 The distribution has been similar in most respects at 1, 6, and 24 hours. High concentrations were found in the liver, spleen, and bone marrow moderate concentrations in red cells, lungs, kidney, and bone and small amounts in all other tissues 50-70 per cent of the administered lead was found in the liver, 15-25 per cent in bone marrow, and 5-12 per cent in bone. Less than 1 per cent was excreted in the urine in the first 24 hours, and 1-2 per cent was found in the contents of the gut.

3 After intramuscular injection of BAL the urinary excretion of lead was increased to 3-15 per cent of the dose, even when BAL was not given until nineteen hours after the lead, and larger amounts of lead were found also in the contents of the gut

4 Other changes when BAL was administered were a reduction of the concentration of lead in the liver, spleen, bone marrow, and red cells, and an increase of the concentration in cardiac and skeletal muscle After BAL 20-48 per cent (in one case 60 per cent) of the lead was found in the liver, 7-13 per cent (in one case 30 per cent) in the bone marrow, 1-13 per cent in bone, and up to 10 per cent in skeletal muscle

5 The significance of these changes is discussed

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THE EFFECT ON GASTRIC SECRETION OF DIFFERENT RATES OF HISTAMINE INFUSION AND OF "NEOANTERGAN"

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In acute experiments on lightly anaesthetized cats, a quantitative study has been made of the gastric secretory response to continuous intravenous infu-This is a necessary sion of histamine solutions preliminary to the use of the method in the investigation of the effects of various substances on gastric The use of an infusion of a low concentration of histamine as a gastric secretory stimulant is not new Ivy and Javois (1924), Gutowski (1924), Teorell (1932, 1933), Emmelin et al (1941), Uvnas (1943), Emmelin (1945), Emmelin and Frost (1947), and Obrink (1946) have reported experiments using this method However, the earlier workers did not maintain a constant rate of infusion for more than a short period Emmelin (1945) used a "special device" and Obrink (1946) used the apparatus described by Lindgren (1943) for the continuous delivery at a constant rate of small volumes of solution from a syringe The apparatus used in the present work is described below

Information was sought on the possibility of maintaining, for some hours, a steady output of gastric juice and acid in the anaesthetized cat was also desired to find out what rate of histamine infusion would give a submaximal stimulation of the secretory cells, such as would be necessary if the method were to be useful for the investigation or assay of inhibitory substances Some guidance was given by the results of previous workers Figures for infusion rates have been calculated approximately from data given in their publications and are presented for convenience in Table I The rate of infusion is given in terms of µg of histamine base per kg of body weight per minute Where the weight of the animal was not recorded (marked *), average weights of 10 and 3 kg have been arbitrarily assumed for dog and cat respectively

TABLE I

Source	Anımal	Anaesthetic	Infusion µg / kg /min	Remarks
Gutowski (1924)	dog*	decerebrate	2	
Ivy and Javois (1924)	pouch dog	none	2 7	submaxımal (30–45 mın)
Teorell (1933)	cat*	chloralose and urethane	22 (approx	submaximal (15 min)
Emmelin et al (1941)	cat pouch dog	decerebrate none	0 5-1 5 0 5-0 6	
Bjorkman et al (1943)	cat	chloralose and urethane	5 1 1 7	maxımıl submıximal
Obrink (1946)	pouch dog	none	22	maxımal
Emmelin and Frost (1947)	cat	chloralose	2-7 usually 2-3	
	<u>' </u>			

From all these reports it seemed likely that an infusion rate of about 2 to 3 µg histamine base per kg of body weight per minute would stimulate a submaximal gastric secretion in most anaesthetized cats

The part played by histamine as a natural gastric secretory stimulant under normal and pathological conditions in man is still undecided, however, the fact that histamine can stimulate gastric secretion has led many workers to investigate the action of antihistamine substances on this response to histamine. Such substances might be useful in the investigation of the exact role of histamine in normal gastric secretion, apart from a possible therapeutic application. There have been conflicting reports on the effects of such substances on gastric secretion, and the position has recently been summarized in the review by Loew (1947). Present

opinion (Grossman and Ivy, 1946, Loew, 1947) is that none of the antihistamine substances so far tested has any significant inhibitory effect on histamine-induced gastric secretion

The potent antihistamine substance, β -dimethylaminoethyl-N-p-methoxybenzyl- α -aminopyridine ("neoantergan," 2786 RP), has recently been introduced in this country as "anthisan" Bovet and Walthert (1944) reported that it did not inhibit gastric secretion induced by histamine in the rat, and this was confirmed in man by Decourt (1945)

Some experiments have been made, using the method described below, to study the effect of neoantergan on the gastric secretory effect of histamine in the anaesthetized cat

EXPERIMENTAL METHODS

Cats weighing from 1 75 to 4 72 kg (average, 2 67 kg) were used after preliminary withholding of solid food for 18 to 24 hours In a few animals anaesthesia was induced with chloroform and continued with cyclopropane and The depth of anaesthesia could be readily oxygen varied from complete surgical anaesthesia during the operative procedure to a light plane of narcosis during the remainder of the experiment. The main disadvantage apart from the slight danger of explosion is that of expense In most animals anaesthesia was produced by intraperitoneal injection of sodium pentobarbitone, small maintenance doses being given intravenously when required later The anaesthetized animal was prepared for collection of gastric secretion by the method of Lim (1923) as recently modified by Roth and Ivy (1944)

The stomach and duodenum are approached by a midline abdominal incision and a cannula is inserted into the stomach through the pylorus from an opening in the first part of the duodenum The duodenum is ligated just proximal to the entry of the bile duct and the cardio-oesophageal junction is also tied, the vagi being excluded from the ligature The cannula is a piece of perforated soft rubber tubing about 7 cm long and of about 0.4 cm bore. It is tied on to a short length of glass tubing around which the pyloric ligature is tied, the local blood vessels being carefully preserved From the other end of the glass tubing another short rubber tube passes to the exterior through a stab wound in the right side of the abdominal wall, at the level of the pylorus The animal is arranged slightly tilted on its right side so that the gastric juice flows readily from the stomach, without stasis In only two of some seventy animals have more than a few drops (<0.5 ml) been found in the stomach at the end of 6-7 hours of collection of juice The stomach is gently rinsed out with warm saline via the pyloric cannula, and after closure of the abdominal incision the animal is left for an hour before the infusion is commenced. The whole procedure takes only a few minutes, and under the conditions described the rate of secretion at the end of one hour is basal and of the order of one or two drops in ten minutes, no free

acid being detected It is important that the animal be kept warm during the whole of the experiment

The infusion apparatus was designed and made by Dr E H J Schuster, it incorporates a cylinder with close-fitting reversible piston, originally a small hydraulic lack This apparatus delivers a constant slow flow of liquid paraffin to a vessel from which the infusion fluid is displaced into the cat By means of an electric gramo phone motor and a variable reversible gear, a continuous infusion can be maintained indefinitely at a rate variable from 0 19 to 6 0 ml per min Histamine acid phosphate is made up in normal saline, and the infusion of 0.75 ml of saline per min easily replaces the fluid and chloride lost in the gastric juice - (Throughout this paper doses of histamine are doses of the base) In control animals infusion of saline alone did not cause any secretion greater than the basal rate The total volume of juice and the amounts of free and total acid were measured. samples being collected at intervals of 10 to 30 minutes for up to 7 hours For acid titration N/50 or N/20sodium hydroxide was used with thymol blue as the usual indicator for the two endpoints Peptic activity of the juice has not been measured.

In most of the experiments with neoantergan it was infused with histamine in the same solution. In three other experiments, after an initial period of two hours during which $5~\mu g$ histamine per min was infused, the infusion was rapidly changed to a mixture of $5~\mu g$ histamine and $15~\mu g$ neoantergan per min for $1\frac{1}{2}$ to 2 hours. Finally the original solution of histamine was substituted for the mixture. The solutions were changed with only a momentary interruption of the flow since it was arranged that the pump could deliver liquid paraffin to either of two bottles by changing a number of clips

RESULTS

The average volumes of juice and of free acid secreted by groups of 6 or 7 cats during successive 30-min periods of histamine infusion are shown in The four groups of cats received histamine infusions of 25, 5, 10, and 20 µg per min respectively for 5-6 hours After an initial delay varying from about 30 to 90 minutes, during which there is a steady increase in secretion, a reasonably constant rate of secretion is maintained for some 5 hours at least. The initial delay is less at the higher rates of infusion, as might be expected The groups receiving 10 and 20 µg histamine per min achieve much the same peak rate of secretion When the average hourly secretion rates are compared with the rate of infusion as in Table II and Fig 2, it can be seen that although there is a wide variation between the responses of individual cats there is a relation between the mean rate of secretion in a group of cats and the rate of histamine infusion It is certain that in most cats secretion is not maximally stimulated by an infusion of 5 µg histamine per min and probably not by 10 µg per min If the rates

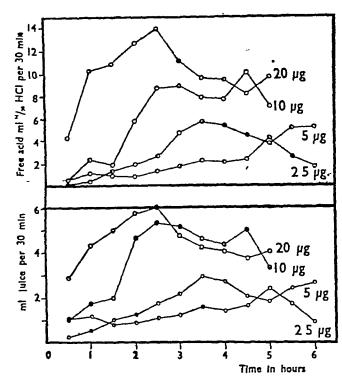


Fig 1—Mean volumes of juice and free acid secreted in successive 30-minute periods by groups of cats receiving different rates of histamine infusion 2 5, 5, 10, and 20 μg/min (equivalent to range of 0 74 to 1.25, 1 4 to 2 3, 3 4 to 4 5, and 5 3 to 9 8 μg histamine/kg/min) in groups of 6, 7, 6, and 6 cats respectively Infusion started at zero time Abscissae, time in hours Ordinates ml, juice and N/20 HCl in 30 min

of infusion are correlated with the weights of the individual animals it is found that a rate of histamine infusion in excess of 4 μg per kg per min causes maximal secretion in most animals. The secretion due to 2 to 3 μg histamine per kg per min is usually submaximal

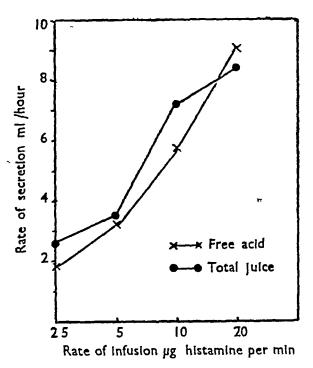


Fig 2—Average hourly secretion rates (gastric juice and free acid) over a period of 5–6 hours in groups of cats, at different rates of histamine infusion Abscissae rate of histamine infusion (logarithmic scale) Ordinates rate of secretion

Infusion of neoantergan alone (15 μg /min) did not stimulate gastric secretion in excess of the "basal" level and no free acid was detected in such juice In Figs 3 and 4 average secretion curves are compared for groups of cats given histamine alone and mixtures of histamine and neoantergan. Secretion in a group of cats given 5 μg histamine and 15 μg neoantergan per min is greater than that due to 5 μg histamine per min alone (Fig 3). This is also apparent from the average hourly secretion rates shown in Table III. From Fig 4 it will be seen that

TABLE II

MEAN RATES OF SECRETION OF JUICE AND FREE ACID IN RELATION TO RATE OF HISTAMINE INFUSION

(Histamine doses are given in terms of free base, the acid phosphate was used)

Group rate of	No of cats	Avge wt	Range of individual infusion rates	Mean rate of secretion of juice		Mean rate of acid production (N/10 HCl)			
infusion	Cats	(kg)	μg /kg /min	ml/hr	σ	€	ml/hr	σ	€
2 5 μg /min	6	27	0 74–1 25	2 61	1 78	0 73	1 89	2 24	1 00
5 0 μg /min	7	2 85	14-23	3 50	2 25	0 85	3 26	3 33 1	1 26
10 0 μg /min	6	2 52	34-45	7 20	3 50	1 43	5 78	3 19	1 30
20 0 μg /min	6	2 58	5 3 -9 8	8 42	2 41	0 98	9 07	4 47	1 83

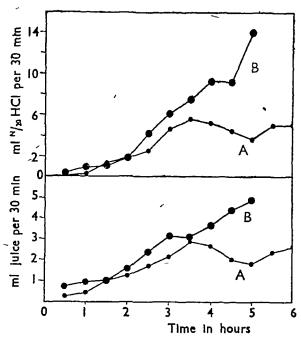


Fig 3—Effect of neoantergan Comparison of secretion of gastric juice and free acid due to 5 µg histamine/min (A) and a mixture of 15 µg neoantergan and 5 µg histamine/min (B) Secretion induced by the mixture is greater than with histamine alone Figures are average values for groups of 7 and 6 cats respectively

TABLE III
THE EFFECT OF NEOANTERGAN ON GASTRIC SECRETION INDUCED BY HISTAMINE INFUSION, 5 μ G/MIN

Infusion $5 \mu g$ histamine per min

- Cat	Walaht	Dunation	Average rate	of secretion
No	Weight kg	Duration, hours	Juice, ml/hr	N/10 acid, ml /hr
28 34 35 36 37 45 58	2 75 3 25 2 80 2 50 2 90 2 18 3 60	6 6 5 6 6 7	5 90 1 20 5 20 0 65 1 90 3 75 5 90	5 85 0 50 6 65 - 0 00 0 65 3 85 5 30
Mean ra	ite ± stan	dard error	3 50 ± 0 85	3 26 ± 1 26
B Infus	sion 5 µg h	istamine +	15 μg neoanter	gan per min
29 40 43 44 47 48	2 50 2 45 1 87 1 75 2 50 3 48	6 5 4 5 6 5	3 10 3 95 - 5 50 6 20 12 05 1 80	2 50 0 75 5 55 6 85 15 55 0 80
Mean ra	ate ± stan	dard error	5 43 ± 1 47	5 33 ± 2 08

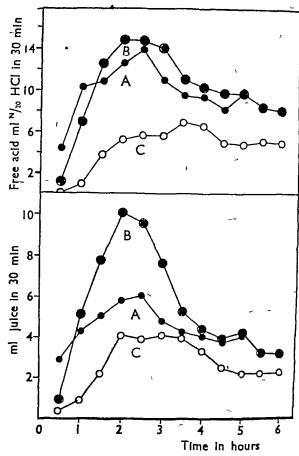


Fig 4—Effect of neoantergan Comparison of average secretion curves for juice and free HCl due to 20 µg histamine per min (6 cats) (A) with that due to infusion of the same amount of histamine and 5 or 15 µg neoantergan per min (3 cats each) 15 µg neoantergan (B), 5 µg neoantergan (C)

the secretion produced by 20 μg histamine and 15 μg neoantergan per min is probably greater than that due to histamine alone, although the secretion produced by 20 μg histamine and 5 μg neoantergan per min is less than that due to histamine alone These contrary findings may be partly due to the small number of animals tested in this group (Table IV)

In two of three other experiments, in each of which the effects of infusions of histamine and neoantergan and of histamine alone were compared in the same animal, there was a definite increase in secretion of juice and acid during the period of the mixed infusion compared with that due to histamine only (Fig 5) The effect was absent in the third cat Certainly no inhibition of gastric secretion was observed

TABLÈ IV
THE EFFECT OF NEOANTERGAN ON GASTRIC SECRETION INDUCED BY 20 µG HISTAMINE/MIN

	20 μg histamine per min'						
	20	rg mstamm	le ber um				
Cat	Wordht	Duration	Average rate	of secretion			
No	Weight kg	Duration, hours	Juice, ml/hr	N/10 HCl, ml/hr			
15 16 17 18 20 64	2 05 2 50 2 30 2 45 2 35 3 80	4 5 6 6 7 5	3 65 9 10 9 20 9 85 10 15 8 50	1 05 9 80 8 85 10 05 14 80 9 90			
Me	an rate ±	error	8 41 ± 0 99	9 08 ± 1 83			
20 μ	g histamii	ne and 5 µg	neoantergan]	per min			
22 23 24	2 50 3 50 2 75	6 6 6	4 35 2 80 8 85	3 50 1 50 9 05			
Mea	n rate ±	error	5 33 ± 1 82	4 67 ± 2 24			
20 /	ug histami	ne and 15 μ	g neoantergar	per min			
25 26 27	1 80 1 80 2 70	6 6 6	11 80 12 90 7 70	2 15 19 20 8 95			
Mea	ın rate ±	etror	108 ± 158	10 1 ± 4 95			

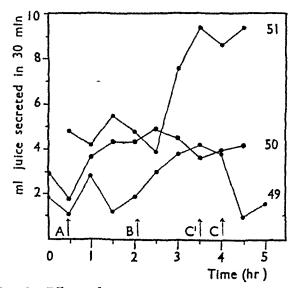


Fig 5—Effect of neoantergan on gastric secretion of cats 49, 50, and 51 5 μg histamine per min at A and C (C' for cat 51), 5 μg histamine and 15 μg neoantergan per min between B and C (C' for cat 51) Pentobarbitone anaesthesia.

The majority of the results suggest the probability that neoantergan may increase rather than reduce the stimulation of gastric secretion by histamine

DISCUSSION

Continuous intravenous infusion of histamine causing submaximal secretion of gastric juice and acid has given in most animals a reasonably steady flow of juice and acid for some hours after the first 30 to 90 minutes, during this initial period the secretion is increasing to the steady level. At the highest rates of infusion used there is a tendency for the rate of secretion, which is probably maximal, to fall off after 5-6 hours In most animals a rate of infusion of 2 to 3 µg histamine per kg per min will not stimulate gastric secretion maximally, and this rate of infusion is probably suitable if it is desired to test the effect of other substances on the secretion produced by histamine In a particular animal one can test whether the secretion is in fact submaximal by observing the effect of increasing the rate of infusion once a steady rate of secretion has been obtained. The former level of secretion is usually regained within 30 minutes of reducing the infusion rate again

The effect of substances which antagonize histamine or histamine-induced gastric secretion can be investigated. The test substance may be injected in single doses during the histamine infusion, or average secretion curves for groups of animals receiving histamine alone can be compared with similar curves for groups receiving histamine and the antagonist. A quantitative estimation of the effect can be obtained with either method

The finding that neoantergan did not inhibit the effect of histamine on gastric secretion was expected in view of earlier work. There seems little doubt that, like other less potent antihistamine agents, neoantergan does not decrease the gastric secretory effect of histamine Even the much more potent thiodiphenylamine derivative, 3277 R P, has been shown to be ineffective against the gastric ulceration produced by large doses of histamine in the guineapig, although protecting the animal from the immediately lethal effect of the histamine (Halpern and Martin, 1946) Clinical evidence confirms that neoantergan has no value in the control of gastric hypersecretion (Decourt, 1945) The evidence reported here strongly suggests that negantergan may actually increase the gastric secretory response to injected histamine Similar effects after the administration of benadryl have been reported by Emmelin and Frost (1947) in anaesthetized cats and by McElin and Horton (1946) and Doran (1947) in man

This probable potentiation of histamine action on gastric secretion may link up with the reported aggravation of symptoms in some asthmatic patients treated with antihistamine substances (see Bovet and Walthert, 1944, p 38) These authors pointed out that this finding might be due to an increased "histaminaemia," and Geiringer (1947) has recently reiterated this possibility in a comment on the conclusions of Doran Proof of such an explanation of the apparently anomalous finding awaits further experiment.

SUMMARY

- 1 Gastric secretion has been collected from cats, anaesthetized with sodium pentobarbitone, by a cannula tied into the stomach through the pylorus Volumes of juice and of free and total acid secreted have been measured
- 2 The mean rate of secretion of juice and of acid in a group of cats is related to the rate of infusion of histamine, although the rate of secretion obtained with a certain rate of histamine infusion varies considerably from animal to animal
- 3 The infusion of 2 to 3 µg histamine per kg per min causes a submaximal secretion in most cats, which remains relatively steady after the first 30 to 90 minutes for at least 5 or 6 hours
- 4 The method can be used to investigate the effect of other substances on the gastric secretion induced by histamine
- 5 The antihistamine substance neoantergan has been found to increase rather than to inhibit the gastric secretion induced by an infusion of histamine The implications of this finding are discussed

These experiments were begun in the Department of Pharmacology, Oxford, at the suggestion of Prof J H Burn To him and to Prof E J Wayne I am grateful for continued stimulation and interest Neoantergan and anthisan were kindly supplied by Dr D Bovet and by Dr R Wien Part of the expenses have been met by a grant from the Medical Research Council

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THE PHARMACOLOGICAL PROPERTIES OF CONESSINE, ISOCONESSINE AND NEOCONESSINE

. BY

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Conessine is an alkaloid obtained from the bark and seeds of *Holarrhena antidysenterica*, a tree growing in India The alkaloid has been studied by many workers (Keidel, 1878, Burn, 1914, Chopra, Gupta, David and Ghosh, 1927, Chopra, 1933, Bakhsh, 1936) who have described various properties bearing little or no relation to one another Conessine is a typical example of the many alkaloids described in reference books of pharmacology the properties of which are difficult to remember because there is no sign of connexion between them and because they appear to be entirely fortuitous

A report from Frère Just. Gillet, S J, missionary in the Belgian Congo, that the chewing of the leaves of the plant Holarrhena congolensis produced anaesthesia of the mucous membrane of the mouth, led Burn (1914) to examine conessine experimentally for local anaesthetic action, which he found it to possess Further work on this action was carried out by Trevan and Boock (1927) The use of conessine as a local anaesthetic is, however, limited by the fact that when injected it causes necrosis Two isomers of conessine, namely isoconessine and neoconessine, have been prepared by Dr S Siddiqui by treating conessine with sulphuric acid Samples of conessine dihydrochloride, isoconessine hydrochloride and neoconessine hydrochloride were left with Prof Burn by Dr Siddiqui with the request that they should be compared with one another for local anaesthetic potency and for local irritant action There was a possibility that one of these substances would prove as potent as conessine and not cause an inflammatory reaction when injected

The three substances have therefore been compared for their irritant action, and the method used for making this comparison will be described in a later paper They have also been compared for local anaesthetic action In addition these substances have been examined for other properties, several local anaesthetics are known to depress the action of acetylcholine on the rectus muscle of the frog, and de Elío (1948) has even observed a quantitative parallelism work (1946) showed that many local anaesthetics had a quinidine-like action on the heart essine and its isomers have therefore been examined on skeletal and on cardiac muscle. Since the results indicated a similarity to quinidine, and since de Elío (1948) had shown that quinidine had a spasmolytic action, reducing the action of acetylcholine on the intestine, conessine and its isomers have also been tested in this way

EXPERIMENTAL OBSERVATIONS

Local anaesthetic action—No figures exist for the relation between the local anaesthetic potency of procaine and conessine when given by injection Conessine and its derivatives were therefore compared with procaine by intracutaneous injection into guinea-pigs according to the method of Bulbring and Wajda (1945) The results for conessine when compared with neoconessine and isoconessine on the same animals are shown in Fig 1, from which it appears that neoconessine is the weal est, having 48 per cent of the strength of conessine, and isoconessine is intermediate, having 77 per cent of the strength of conessine. A careful comparison of

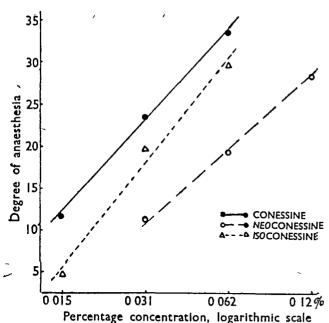


Fig 1—Graph showing the relative local anaesthetic potencies of conessine, isoconessine and neoconessine Each point represents the mean degree of anaesthesia in 8 areas If conessine = 100, then isoconessine = 77 and neoconessine = 48

TABLE I

LOCAL ANAESTHETIC POTENCIES IN TERMS OF CONESSINE
= 100

(Intracutaneous injection into guinea-pigs)

	Local anaesthetic action	Inhibition of ACh on frog rectus
Conessine Isoconessine Neoconessine Cocaine Quinidine Procaine	100 77 48 47 10 7	100 73 60 30 20 10

conessine with procaine showed that the relative anaesthetic potencies can be expressed as in Table I, in which the figures for quinidine (de Elio, 1948) and for cocaine (Billbring and Wajda, 1945) are also included for comparison

Table I shows that conessine is appreciably stronger than its derivatives, but that the weakest, neoconessine, differs little in potency from cocaine

Frog rectus—The stimulant action of acetylcholine on the frog rectus muscle was found to be depressed by conessine and its isomers. An illustration of this action is given-in Fig 2 in which the effects of all three substances are shown. A direct comparison of isoconessine and quinidine

showed that quinidine had 28 per cent of the potency of *iso* conessine in depressing acetylcholine on the frog rectus When de Elío (1948) compared four local anaesthetics, procaine, cocaine, ametho-

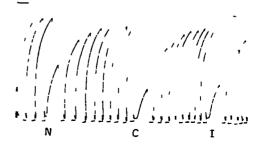


Fig 2—Record of contractions of the frog rectus abdominis produced by a concentration of 3.75 × 10⁻⁷ acetylcholine acting for 90 sec. The contractions were obtained at 6 min intervals and those marked N, C and I were preceded by exposure of the muscle for 4½ min to concentrations of 4 × 10⁻⁵ neckonessine, conessine and isoconessine respectively. If conessine = 100, 'then isoconessine = 73 and neckonessine = 60

caine and nupercaine, he found that they likewise depressed the action of acetylcholine on the frog rectus and moreover that their relative potency in doing so was similar to their relative potency as local anaesthetics. This was also true for conessine and its isomers as shown in Table I, in which the two sets of figures are placed side by side.

Denervated gastrocnemius of cat -Similar experiments were also carried out on the denervated gastrocnemius muscle of the cat The left sciatic nerves of a series of cats were divided under ether with aseptic precautions (these operations were kindly performed by Prof Burn) and the animals were left for 5-26 days The cats were then anaesthetized with ether and chloralose and the gastrocnemius muscle was detached from the os calcis and fastened to a tension lever Injections were made through a cannula in the right iliac artery pointing towards the bifurcation of the aorta Contractions were then obtained by the injection of acetylcholine, and the effect of a preceding injection of conessine on these contractions An illustration of two experiwas determined ments is given in Fig 3 The usual result was that injections from 1-10 mg depressed the response In the upper part of Fig 3 this depression is shown produced by 10 mg isoconessine and by 10 mg conessine In the lower part of Fig 3 a less usual effect is shown, namely, that 1 mg of these same alkaloids produced an augmentation of the acetylcholine response

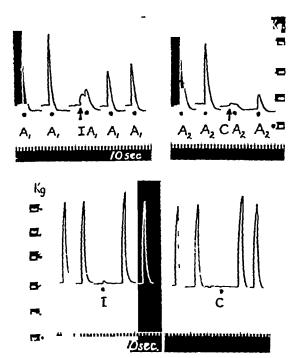


Fig 3 —Cat. Chloralose Record of contraction of denervated gastrocnemius in response to intraarterial injections of acetylcholine

Upper tracing Muscle denervated 27 days before Contractions at A_1 due to 10 μg ACh Intraarterial injection of 10 mg isoconessine produced a small contraction, and the following contraction in response to ACh was greatly reduced Contraction at A_2 produced by 20 μg ACh 10 mg conessine abolished the effect of the next injection of ACh

Lower tracing Muscle denervated 26 days before Contractions produced by 10 µg ACh. The intraarterial injection of 1 mg isoconessine at I, and of 1 mg conessine at C, slightly increased the effect of the next injections of ACh.

Rat diaphragin preparation—Since the work of Harvey (1939) it has been known that quinine has various actions on skeletal muscle among which are (1) the ability to increase the tension response to a single maximal stimulus, and (2) to act like curare in lowering the excitability at the motor end plate Experiments were therefore carried out to see if these effects could be demonstrated when conessine and its isomers were used. It was found that the curare-like action was regularly demonstrable, but only once was the augmentor action seen isolated nerve-muscle preparation of the rat as described by Bulbring (1946) was used The effect is shown in Fig 4 in which the phrenic nerve was stimulated by maximal single shocks, in the upper part of the figure the addition of 0.5 mg isoconessine to the bath caused an increase in the response very similar to that seen in the lower part of the figure when 2 mg quinidine was added. The usual response to the addition of isoconessine was

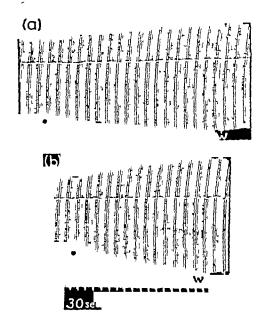
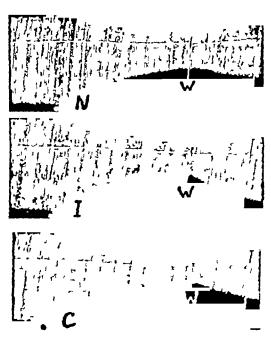


Fig 4—Rat diaphragm phrenic nerve preparation Tyrode 37° C, bath 50 ml Record of the contractions produced by stimulating the nerve with single maximal shocks 8–10 times per min At (a) is shown the increase in amplitude produced by 0.5 mg isoconessine. This is an unusual effect. At (b) is shown the action of 2 mg quinidine.



Record of maximal contractions. Depression of contraction produced by 1 mg neoconessine at 1 mg isoconessine at 1 and 1 mg conessine at C. All three substances were in the bath for 10 m n before washing out. The effects were reversible

that shown in Fig 5 in which the muscle twitches were steadily reduced as they are when d-tubocurarine is added, the same result was obtained with conessine and neoconessine. The doses used were 5-10 times as great as the dose of d-tubocurarine which would have been required to produce a similar effect.

Quinidine-like action on cardiac tissue—The alkaloids were tested by Dawes's method (1946) in which the isolated rabbit auricles are suspended in

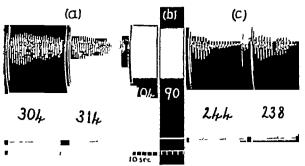


Fig 6—Isolated rabbit auricle driven electrically Ringer-Locke at 29° C, bath 50 ml (a) Auricle follows 304 but does not follow 314 stimuli per min Spontaneous rate 104 beats per min (b) Spontaneous rate after 5 min exposure to 4 × 10⁻⁶ isoconessine (c) After 10 min exposure to isoconessine the auricle fails to follow 244 but follows 238 stimuli per min

a bath at 29° C and driven electrically The maximum rate at which the auricles will follow the electrical stimulus is determined before, and then after exposure for 10 min to a given concentration of the substance When quinidine is tested in this way-it reduces the maximum rate, and this reduction persists for some time after the quinidine has been washed out. However, provided an interval is allowed, the preparation returns to its former state and further observations can then be When conessine or its isomers were used. a similar reduction in the maximum rate was observed (see Fig 6), but the effect of a given concentration on different occasions was not the same, and often an increase in concentration produced little increase in effect. From the best experiments it appeared that conessine in a concentration of 2×10^{-5} was approximately equal to aumidine 10 5 Thus the potency of conessine was 50 per cent of that of quinidine Isoconessine was weaker than this

Rabbit auricles — Acetylcholine causes diminution of the spontaneous contractions of the isolated rabbit auricles, as shown in Fig 7, in which the effect of 50 μ g acetylcholine is shown twice at the beginning of the record. After washing out, when the contractions had recovered their original size, 2 mg isoconessine was added to the bath, and 1 min later 50 μ g acetylcholine was added again



Fig 7—Isolated rabbit auricle beating freely small arrows †, 50 µg ACh was added to the bath and was washed out when the drum was stopped at the reversed arrows ‡ 2 mg. isoconessine was added to the bath at the large arrow (1 min before the next dose of ACh) and remained in the bath during the period shown by the horizontal line. The inhibitory action of ACh was abolished and had not returned 10 min after washing-the isoconessine from the bath. The apparent augmentation by ACh in the presence of isoconessine was believed to be an artefact.

No diminution was seen, either then, or at a further addition of acetylcholine after the isoconessine was washed out. After further washing out, the inhibitory effect of acetylcholine slowly returned, though not fully. Both conessine and neoconessine had the same effect.

Action on the heart—In the rabbit heart perfused by Langendorff's method, the alkaloids were observed to have a dilator action on the coronary vessels which was most marked with neoconessine,

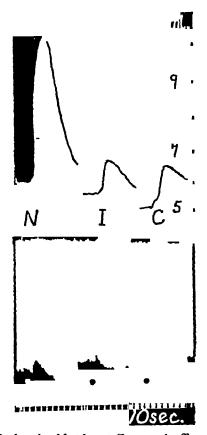


Fig 8—Isolated rabbit heart (Langendorff preparation)
Perfused with Ringer-Locke at 37° C Record from above downward Coronary flow ml per min, amplitude of heart beats and time marker At N, I and C 200 µg of neoconessine, isoconessine and conessine respectively were injected into the perfusion fluid Note large dilator effect of neoconessine (For method of recording, see Stephenson, 1948)

and sometimes absent with conessine. A comparison of the dilator effect of the three substances is shown in Fig. 8, where they are also seen to cause a slight increase in amplitude of the beat. Bakhsh (1936) noted the dilator action of isoconessine on the coronary vessels.

Action on blood vessels—To test the effect on the vessels both the rat's hindlegs and the rabbit's ear have been used, perfused with Ringer's solution

at room temperature The rat's hindlegs were perfused through the abdominal aorta and the rabbit ear by the method of Gaddum and Kwiatkowski (1938) Conessine, when injected into the fluid perfusing the rabbit's ear, caused vasodilatation It was curious to find that whereas the constrictor effect of an injection of adrenaline in the rabbit's ear was greatly reduced by an injection of isoconessine, the constrictor effect of adrenaline in the rat's hindlegs was not affected. The difference was perhaps more apparent than real, since when an adrenaline tone was maintained in the rat's hindlegs, the injection of conessine (0.1 mg) caused dilatation (Fig. 9a) In the rat's hindlegs, a single injection of acetylcholine during the maintenance of an adrenaline tone caused dilatation not only adrenaline but also conessine was present

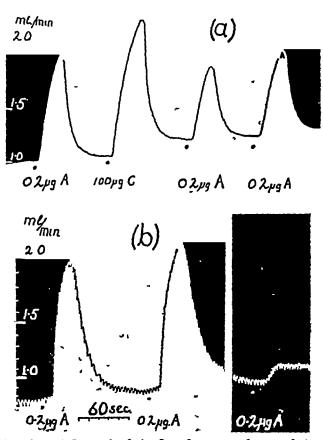


Fig 9—(a) Record of the flow from a perfusion of the hindlegs of a rat with Ringer-Locke at room temperature. The perfusion contained 10° adrenaline and dilatations were produced by 0.2 · g. ACh and by 100 µg conessine. The effect of ACh is reduced after conessine. The record is continuous and the injections were made at 10 min intervals. (b) Dilatations were produced by 0.2 · g. ACh. In the second part, the perfusion fluid contained 10 · conessine in addition to adrenaline. Note that in the presence of conessine, the dilator effect of ACh was greatly reduced.

in the perfusing fluid, the dilator action of acetylcholine was almost abolished (Fig 9b)

Action on blood pressure and respiration—The actions of neoconessine and isoconessine were found to be indistinguishable from that of conessine on the blood pressure

It was shown by Burn (1914) that a large dose of conessine produces a fall of blood pressure in

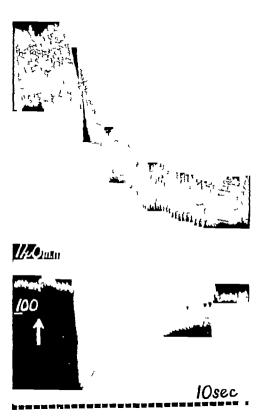


Fig 10—Rabbit, urethane Record of respiration (above) and blood pressure At the arrow 8 mg conessine was injected After about 1 min this produced a sudden slowing of the heart rate accompanied by a fall of pressure The pressure slowly recovered and the heart block suddenly disappeared The respiration was depressed and recovered very slowly and incompletely

which the heart action at first is unchanged. The fall is presumably due to the vasodilatation which has been described above. After some delay the heart is affected, the ventricular rate becoming very slow, the heart then appears to be in block. When smaller doses are used, either or both of these effects may be seen. In Fig. 10 when 8 mg conessine was injected there was no initial fall, for vasodilatation did not occur, after a delay of

1 min the heart became very slow and the pressure fell Gradually the pressure rose again until suddenly the block disappeared and the blood pressure was fully restored

Bakhsh (1936) suggested that the cardiac slowing was central in origin, but since conessine depressed the action of acetylcholine in so many directions it seemed unlikely that this would be so The effect of conessine on vagal stimulation was therefore tested. In the experiment illustrated in Fig. 11 the injection of 8 mg conessine abolished the fall of-blood pressure produced by vagal stimulation, and diminished the depressor effect of 20 µg acetylcholine by cutting out the slowing of the heart which this dose produced. When the respiration was recorded by Gaddum's method (1941), 8 mg conessine was observed to diminish both rate and depth (see Fig. 10)

It is worth while to record the great tolerance of rabbits to the intravenous infusion of isoconessine at a uniform rate. No less than 106 mg was infused into one rabbit, the heart continuing to beat strongly, only when the rate of infusion exceeded 25 mg per min was the heart seriously affected. Solmann (1942) states that quinidine leaves the blood stream very rapidly, and it may be that isoconessine does so also

Spasmolytic action —The spasmolytic action of four local anaesthetics and of quinidine was compared with that of atropine by de Elío (1948) He found that quinidine was six times more powerful than procaine, and procaine was equal to cocaine Conessine has been stated both to relax and to stimulate smooth muscle but no one has hitherto shown that it depresses the action of acetylcholine Fig 12 is a record of the contractions of an isolated piece of rabbit intestine, in which it will be seen that in the presence of concentrations of conessine from 10^{-5} to 2×10^{6} , the stimulant action of acetylcholine is proportionally reduced Both neoconessine and isoconessine were found to be slightly stronger than conessine, and about equal to one another When a comparison was made between conessine and quinidine on a loop of guinea-pig ileum, it was found that conessine was slightly less potent than quinidine

Antimalarial action—In view of the close similarity of conessine and its isomers to quinine and quinidine, a comparison was made with quinine in chickens infected with Plasmodium gallinaceum. This test was done under the supervision of Mr L G Goodwin in the Wellcome Research Institute. Chicks, 6 days old, were inoculated intravenously with 0.2 ml blood



Fig 11—Rabbit, urethane Record of blood pressure At A 20 µg ACh were injected intravenously, at B 1 µg ACh was injected and at V the peripheral end of the right vagus was stimulated for 15 sec. At the arrow, 8 mg conessine was injected and this abolished the effect of the vagus on the heart. The heart was no longer affected by 20 µg ACh, though the injection still produced a fall of pressure After 5 min the heart was again sensitive to vagal stimulation but the effect was not as great as previously

diluted to contain 10⁸ parasitized red cells. The alkaloids were given by mouth, once on the day of inoculation, and twice on each of the three following days, 17 hours after the last dose, a blood smear was prepared from each bird in which 200 red cells were examined to determine the percentage which contained parasites. The results, recorded in Table II, indicate that, in the doses used, no antimalarial effect was exerted by conessine or its isomers.

TABLE II

ANTIMALARIAL ACTION IN CHICKS INFECTED WITH

P Gallinaceum

Substance	Dose mg./kg	No of birds	Mean percentage of cells with parasites	
(Controls)		7	42	
Conessine ,,	40 20	4	32 51	
Isoconessine	80 40 20	4 8 8	46 37 42	
Neoconessine	40 20	3 3	38 21	
Quinine	40 20	8 8	0 5 11 4	

Toxicity—Conessine and its isomers were compared with one another by intravenous injection into mice in order to determine the mean lethal dose. Care was taken to inject at a uniform rate. For conessine 39 mice were used, for isoconessine 30 mice, and for neoconessine 50 mice. The lethal dose killing 50 per cent of mice was for conessine 28.7 mg. per kg., for isoconessine 33.2 mg. per

kg, and for neoconessine 13 1 mg per kg. Thus whereas conessine and isoconessine are very similar in toxicity, neoconessine is more than twice as toxic. It may be emphasized that while the actual figure for the toxicity of any one compound may vary greatly in different laboratories, the relative toxicities of the three compounds should remain constant.

DISCUSSION

Recent papers from this laboratory (Dawes, 1946, Dews and Graham, 1946, de Elío, 1948, Dutta, 1948) have brought out the similarity between various substances not previously considered to be related Dawes's work showed that substances with a quinidine-like action on the heart included various local anaesthetics, spasmolytics and analgesics, and that quinidine and procaine reduced the action of acetylcholine in cardiac, skeletal and unstriped muscle De Elio added to the evidence by testing four local anaesthetics, as well as atropine, pethidine and quinidine, on the frog rectus, the rabbit auricles and the rabbit intestine. In general all seven substances reduced the action of acetylcholine in these tissues and a close parallelism between local anaesthetic action and effect on the frog rectus was found to exist. Dutta tested pethidine quinidine, atropine and benadryl to see whether they possessed the property, which procaine has long been known to possess, of lowering body temperature He found that all these substances resembled procaine in this respect

When conessine and its isomers were to be examined, their known local anaesthetic activity suggested that they also should be examined from the point of view described, and that since quinidine was also a plant all aloid with local anaesthetic properties, the relation of their action to

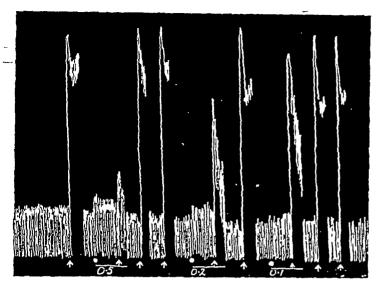


Fig. 12—Isolated rabbit intestine, Ringer-Locke 36° C. The contractions at the small arrows were produced by the addition of 8 μg. ACh to the bath Contractions produced in the presence of 0.5 mg, 0.2 mg. and 0.1 mg of conessine (added at the white spot) were reduced in proportion to the amount of conessine. The fluid in the bath was changed at the end of the horizontal lines.

that of quinidine should be considered with special care. The outcome has been to demonstrate that in many respects conessine and its isomers are very similar to quinine and quinidine. So close indeed did the resemblance seem at one point that tests for anti-malarial activity were carried out. However, in doses in which quinine was active, conessine and its isomers were inactive. They have not yet been tested in higher doses.

Conessine resembles quinidine in diminishing the action of acetylcholine on the isolated intestine, the rabbit auricle, the frog rectus and on denervated mammalian muscle The contractions of the rat diaphragm produced by nerve stimulation are occasionally augmented by isoconessine as they usually are by quinidine, the usual effect of conessine is depression and this is sometimes seen with quinidine Conessine, like quinidine, lengthens the refractory period of cardiac tissue, and the effect of vagal stimulation on the heart the anaesthetized rabbit is temporarily abolished by conessine as by quinidine (Starr, 1936) and by quinine in the dog (Babkin and Ritchie, 1945)

Of considerable interest is the parallelism between local anaesthetic action and inhibition of the action of acetylcholine on the frog rectus. This was first observed by de Elfo, and the observations on conessine and its isomers make the parallelism still more complete. This is a further addition to the evidence that the action of acetylcholine is concerned with the sensation of pain Harvey, Lilienthal and Talbot (1941) found that when acetylcholine was injected intra-arterially in men it caused very severe pain. Gray (1947) has detached a portion of the skin of an anaesthe-

tized cat so as to leave only the artery, vein and nerve in connexion with the body. When he injected acetylcholine into the artery, he found that an action potential was set up in the nerve, and that this action potential was similar to that produced by mechanical pressure on the detached portion of skin. Thus a local anaesthetic may be a substance which reduces the action of acetylcholine at sensory nerve endings, and this would explain why local anaesthetic potency is related to the reduction of the action of acetylcholine on the frog rectus.

Hitherto conessine has been thought to be an alkaloid with its own peculiarities unlike those of any other plant alkaloid. It is now evident that this is not so, and that the action of conessine, which comes from the bark of Holarrhena, is very similar to that of the alkaloids quinidine and quinine which come from the bark of Cinchona The actions of conessine also resemble those of papaverine, cocaine and atropine, three other plant alkaloids, all of which are local anaesthetics, spasmolytics and have a quinidine-like action on the heart. It is an obvious task to look at still more plant alkaloids in order to see how many others can be brought into this class, and to examine them for points of chemical and physical similarity

SUMMARY

1 Conessine and its isomers, isoconessine and neoconessine, have been shown to possess properties very similar to those possessed by quinine and quinidine Conessine, however, has no antimalarial action in chickens when tested in the same dose as quinine

- 2 The local anaesthetic potency of conessine and its isomers is great, when tested by intracutaneous injection into guinea-pigs, conessine is about twice as active as cocaine, isoconessine is 50 per cent stronger than cocaine, and neoconessine is about equal to cocaine. The relative local anaesthetic potencies of conessine, its isomers, cocaine, quinidine and procaine are very similar to their relative activities in depressing the action of acetylcholine on the frog rectus muscle
- 3 Conessine and its isomers have a quinidinelike action on the heart, and depress the action of acetylcholine on skeletal, cardiac and smooth muscle including that of the blood vessels, acting on all tissues very much like quinidine

This work has been done while in receipt of a grant from the Therapeutic Research Corporation, to whom I wish to express my thanks I am also indebted to Dr S Siddiqui for a supply of conessine, isoconessine, and neoconessine The work has been carried out under the direction of Prof J H. Burn, and this paper has been written with his help

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THE ACTION OF SUBSTANCES WHICH ANTAGONIZE ACETYLCHOLINE ON THE BODY TEMPERATURE OF MICE, BEFORE AND AFTER ADRENALECTOMY

BY

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In 1931 Glaubach and Pick described the fall of temperature produced in guinea-pigs by the injection of procaine Recently Peczenik (1947) has extended this observation to castrated mice, and has shown in addition that the fall is greater after adrenalectomy. By administration of desoxy-corticosterone acetate, progesterone, and other steroids before adrenalectomy, the mice were protected to a varying extent, so that the fall of temperature due to procaine was smaller

No light has hitherto been shed on the reason for this action of procaine Peczenik says that the fall is probably the result of a shock to the autonomic nervous system Recently, however, attention has been drawn to other properties possessed by procaine in addition to its local anaesthetic action Dawes's work (1946) on quinidine substitutes led him to point out that quinine, quinidine, and procame reduce the action of acetylcholine on the intestine, on the heart, and, as was shown by Harvey (1939a and b), on skeletal muscle Dawes showed that procaine, atropine, and pethidine act like quinidine on the electrically driven auricle, and having pointed out the local anaesthetic action of atropine, which has long been known, he demonstrated that pethidine possesses a local anaesthetic action too Thus atropine, pethidine, procaine, and quinidine are four substances having several properties in common, and de Elío (1948) has determined their relative potency in reducing the action of acetylcholine on the frog rectus, the rabbit intestine, and on the isolated rabbit auricle question then arose whether atropine, pethidine, and quinidine would also share with procaine the ability to cause a fall of body temperature in mice The experiments here described supply the answer The antihistamine substance benadryl was also included in the investigation because of the observations of Dews and Graham (1946), they tested the antihistamine substance neoantergan, which is known to have some atropine-like action, and found it to possess a quinidine-like action on the auricle, and a local anaesthetic action. I have found that neoantergan also depresses the action of acetylcholine on the frog rectus and the rabbit auricle, though the doses required are rather large Benadryl also has been shown to have a local anaesthetic action (Leavitt and Code, 1947) and to reduce the action of acetylcholine on the intestine (Loew, MacMillan, and Kaiser, 1946)

METHODS

Since Peczenik has described the conditions for observing the fall of temperature produced by procaine in mice, his procedure was followed in several respects. Male mice weighing not less than 26 g were used, and all were castrated. Peczenik states that non-castrated mice 'did not behave uniformly' They had access to food and water except during the observation of body temperature. They were fed on a diet of constant composition consisting of ground grain, its percentage composition was wheat 19, bran 19, oats 14, maize 9, barley 9, fish-meal 5, bonemeal 9, skimmed milk 14, yeast 1, salt 05, and codliver oil 05 per cent. The mice were kept at about 25° C and experiments were carried out at 25° ±1° C in a small room with a thermostat.

The rectal temperature of each mouse was recorded by inserting a thermocouple into the rectum for 2.5 cm, this thermocouple was connected to a moving coil galvanometer, and the other thermocouple was placed in melted sodium sulphate crystals in a thermos flask. The thermocouple in the rectum of the mouse was kept in position by adhesive tape fixed to the tail, and the tail was fixed along its length to a copper wire which continued forwards over the back of the mouse as far as the forelegs, where it turned around the body of the mouse in a ring behind

them, the mouse was thus held comfortably throughout the period of observation and passed faeces as usual

The temperatures of 8 mice were followed in each experiment during the same period of time, 4 mice were used as controls and were injected with saline, while 4 were injected with the substance being tested. The injections of atropine, pethidine, and procaine were subcutaneous, while those of benadryl and quinidine were intraperitoneal. Before each experiment all mice were kept in the room until their temperatures remained steady, this usually required 30-45 min

RESULTS

Pethidine (demerol)—In order to illustrate the performance of the tests, the results with one dose of pethidine will be given in detail so that the remaining results can be given in summarized form

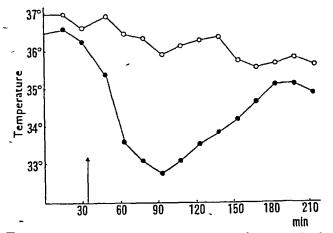


Fig 1—Abscissae time in min, ordinates body temperature in °C White circles, mean body temperature of 4 control mice injected at arrow with saline Black circles, mean body temperature of 4 mice injected at arrow with pethidine hydrochloride, 30 mg/kg

Observations were made on three groups of 8 castrated mice, half of which received 30 mg/kg pethidine hydrochloride, and half received the same volume of saline The results in one of these groups are given in Fig 1 The body temperature of the control mice fell during the 3 hours after the injection from a mean value of 366° C to a mean value of 356° C The temperature of the mice injected with pethidine reached a minimum of 327° C one hour after injection and slowly recovered to 352° during the next 1½ hours results in the other two groups were similar to those shown in Fig 1, they are given in Table I It will be noted that the fall of temperature in the control mice was greater in both groups than that in Fig 1 In Group 2 the mean temperature fell from 355 to 338°, while in Group 3 it fell from 34 6 to 32 9° in the course of the experiment. In

TABLE I
TEMPERATURES OF MICE RECEIVING 30 MG /KG PETHIDINE HYDROCHLORIDE

Time min	Group 2			Group 3		
	Control	Injected	Differ- ence	Control	Injected	Differ- ence
0 15 30 45 60 75 90 105 120 135 150 165	35 5 35 9 35 1 34 7 34 6 34 5 34 4 34 2 34 2 34 2 34 2 34 2	35 5 35 1 33 7 32 6 32 3 32 6 33 1 33 1 33 5 33 6 34 5	0 0 8 1 4 2 1 2 3 1 9 1 3 1 3 0 7 0 6 -0 3 -0 3	34 6 34 6 34 3 34 2 34 3 34 1 33 7 33 7 33 3 33 0 33 1 32 9	35 8 35 0 33 8 33 2 32 9 32 8 32 8 33 0 32 5 32 6 32 6	-1 2 -0 4 0 5 1 0 1 4 1 3 0 9 0 7 0 8 0 5 0 5

Table I the differences between the control mice and those injected with pethidine are also shown The mean differences for the three groups were calculated and the resulting figures are plotted as ordinates against time as abscissa in Fig 2 In this and succeeding Figures, the mean difference of temperature between the two groups of mice before

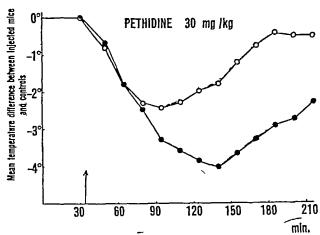


Fig 2—Ordinates difference between mean body temperature of mice injected with 30 mg/kg pethidine hydrochloride and of control mice, taking difference before injection as zero and correcting throughout for this difference. White circles show observations on 12 injected and 12 control mice before adrenalectomy. Black circles show observations on 9 injected and 8 control mice after adrenalectomy.

injection is shown as 0° , this was often not actually so (see Fig. 1), but the difference of temperature between the two groups throughout the period of observation was corrected by the difference which existed before injection

The mice used in obtaining these results were kept for one week after the observations were

made, adrenalectomy was then carried out under ether. Two days after the operation the effect of the same dose of pethidine was redetermined. Of the 24 mice tested before the operation, 7 died during the next two days, so that only 17 were available, of these 8 were injected with saline and 9 were injected with pethidine. The differences of temperature between the mice receiving pethidine and the control mice are recorded in the lower curve of Fig. 2. Fig. 2 shows that after adrenal-ectomy the fall of temperature caused by pethidine was much greater than before

Observations were also made with a higher dose of pethidine, namely 100 mg/kg. In the mice before adrenalectomy this dose produced a rise of body temperature, the mean maximum rise in 12

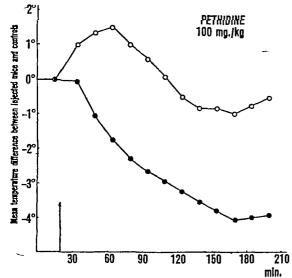


Fig 3 —Similar to Fig 2 Dose of pethidine 100 mg./kg
Note the rise of temperature before adrenalectomy
when 12 mice were compared with 12 controls
Black circles show results after adrenalectomy,
(10 mice injected and 9 controls)

animals being to a point 15° above the mean temperature of the 12 control mice. The rise, however, subsided after 15 hours and was followed by a fall. The change is shown in Fig. 3. This high dose of pethidine not only produced a rise of temperature but also caused increased muscular movements in all the mice.

The rise disappeared after adrenalectomy 10 mice were injected with pethidine and 9 were injected with saline. The pethidine caused a large fall of temperature relative to the temperature of the control mice, this is shown in the lower curve of Fig. 3.

Atropine —Similar experiments were then made with atropine, using first of all 150 mg/kg of

atropine sulphate This dose was too high, in two of the three groups used, the dose was fatal to the mice after adrenalectomy. In one of the groups, however, the tests were carried out successfully, and, though the maximum fall of temperature before adrenalectomy was only 13° more than in the controls, after adrenalectomy the maximum fall was 32° more than in the controls. A further

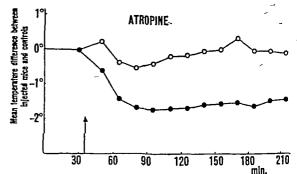


Fig 4—Similar to Fig 2 Mice injected with atropine sulphate 30 mg/kg White circles, 12 injected and 12 control mice before adrenalectomy Black circles, 10 injected and 10 control mice after adrenalectomy

test was then made using 30 mg/kg-atropine sulphate, and the results which were obtained in 24 mice before adrenalectomy and in 20 of these after adrenalectomy are given in Fig 4 Before the operation the fall of temperature was slight, the maximum being 0.5° more than the controls. This is an effect too small to be discovered except by careful investigation. After adrenalectomy it was increased to about 1.5°, and this was maintained.

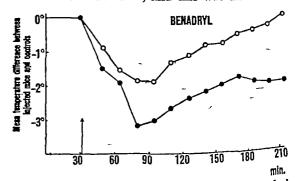


Fig 5—Similar to Fig 2 Mice injected with benadryl 20 mg/kg White circles, 8 injected and 8 control mice before adrenalectomy Black circles, 7 injected and 7 control mice after adrenalectomy

Benadryl —Results with the hydrochloride of β -dimethylaminoethylbenzhydrylether, which is called benadryl, are shown in Fig 5, they were obtained with a dose of 20 mg/kg Before adren-

alectomy 16 mice were used, and after adrenalectomy 14 of the same mice were used. The results were similar to those obtained with the lower dose of pethidine, though the fall of temperature was less.

Procaine—Peczenik injected his mice with procaine hydrochloride in a dose of 100 mg/kg When this amount was used, the fall of temperature was very small, and was not much greater after adrenal ectomy. The maximum fall in 8 mice compared with 8 controls was 05° before and

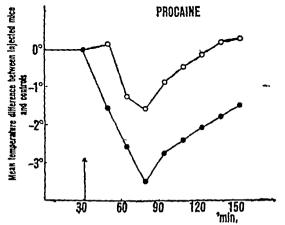


Fig 6—Similar to Fig 2 Mice injected with procaine hydrochloride 200 mg/kg White circles, 8 injected and 8 control mice before adrenalectomy Black circles, 8 injected and 6 control mice after adrenalectomy

15° after the operation. Further observations were then made with 200 mg/kg, and the results in 8 mice, compared with 8 controls, are shown in Fig 6. The difference in sensitiveness between mice in this laboratory and those in Peczenik's laboratory is not surprising, for similar differences are often seen.

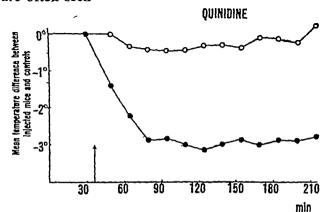


Fig 7—Similar to Fig 2 Mice injected with quinidifie hydrochloride 30 mg/kg White circles, 12 injected and 11 control mice before adrenalectomy Black circles, 9 injected and 7 control mice after adrenalectomy.

Quinidine—The final observations were made when quinidine hydrochloride was given in a dose of 30 mg/kg. As after atropine, there was a very small fall of temperature before adrenalectomy, not exceeding 0.5°. After the operation there was, however, a fall of 3°, which was maintained throughout the period of observation. The results are shown in Fig. 7

DISCUSSION

So far as the action of procaine is concerned. the results described confirm the finding of Peczenik that procaine causes a fall of body temperature in mice and that this fall is greater after adrenalectomy However, the "collapse" of temperature which Peczenik observed after adrenalectomy was not seen, the temperature fell, but so did that of many control mice, and the effect of procaine was not great Peczenik found the mean fall in 20 mice after adrenalectomy to be 49° when procaine was given in the dose 100 mg/kg. he does not record the temperature changes in control mice kept alongside In the work here described, the maximum difference between injected mice and control mice was found to be only 15° after 100 mg/kg, and only 35° after twice this amount The difficulty which a mouse has in maintaining a constant temperature has been discussed by Fuhrman (1946), on account of its large surface per unit weight, the body temperature falls more easily than in larger animals, and control observations are therefore essential

The main result of this research is to demonstrate that the property which procaine possesses of reducing temperature is shared by a number of other substances—atropine, quinidine, pethidine, and benadryl The experiments were undertaken in order to see if this would be so, and the hypothesis concerning their action has thus gained additional support It was pointed out that all these drugs reduce or abolish the action of acetylcholine on the frog rectus, on the spontaneous contractions of the rabbit auricle, and on the rabbit Further, atropine, procaine, and pethidine act like quinidine on the refractory period of the electrically driven auricles of the rabbit (Dawes, 1946) I myself have observed that benadryl reduces the effect of acetylcholine on the frog rectus and the rabbit auricle, and I have found that it lengthens the refractory period Elío (1947) has shown that acetylcholine shortens the refractory period, the quinidine-like action of this group, if not one which can be described as a depression of the action of acetycholine, is at least an action in the opposite direction to that of

acetylcholine Thus there is some ground for the view that the common property of reducing body temperature may be connected with the power to reduce or antagonize the action of acetylcholine

It is at least clear that the common property is not due to the possession of a local anaesthetic action, though all of these substances have one Thus it was found by Glaubach and Pick (1931) that while cocaine caused a varying change of body temperature—namely, a rise in rabbits and a fall in guinea-pigs-nupercaine, one of the most potent local anaesthetics, had almost no effect on the temperature of either species This is of interest because de Elfo found that nupercaine did not reduce the action of acetylcholine on the rabbit auricles when spontaneously contracting, no matter in what dose nupercaine was applied Thus nupercaine provides an example of a local anaesthetic which differs from procaine in reducing neither body temperature nor the action of acetylcholine in the cardiac tissue of the rabbit

The conclusion that substances which reduce body temperature are also substances which reduce the action of acetylcholine must not be taken to imply that acetylcholine plays a role in the central nervous system to maintain body temperature Feitelberg, Pick, and von Warsberg (1939) tested the action of acetylcholine on the temperature of the grey matter of the cortex of cats and found that sometimes it caused a fall and sometimes a rise The fall which procaine, pethidine, etc., cause may be due to their inhibition of the action of acetylcholine in the skeletal muscles, on the activity of which the normal formation of heat depends This suggestion is borne out by the fact that the large dose of pethidine which caused an initial rise of temperature also caused increased motor The lower dose of pethidine, and also the doses of the other substances used, were too small to cause any visible effect in the mice

The effect of adrenalectomy in augmenting the fall of temperature produced by these substances shows clearly that the adrenal glands are concerned in temperature control. Whether it is the cortex or the medulla which is the more important is not known for certain, though the power of cortical extracts to prevent the fall of temperature in adrenalectomized rats when exposed to low temperature indicates the greater importance of the cortex. Moreover, Peczenik claims to have shown

that the administration of desoxycorticosterone acetate diminished the effect of removing the glands on the procaine fall of temperature

A word is required in conclusion on the use of castrated mice. Observations on 16 normal male mice, 8 of which were injected with 30 mg/kg pethidine and 8 of which were controls, gave a result almost the same as that obtained with castrated mice.

SUMMARY

1 The finding that procaine causes a fall of body temperature in mice, and that this fall is greater after adrenal ectomy, has been confirmed

2 It has been shown that this property of procaine is shared by the analgesic pethidine (demerol), by quinidine, by atropine, and by the antihistamine substance benadryl

3 The fall of temperature produced by each of these substances is augmented by adrenalectomy

- 4 All these substances reduce or abolish the action of acetylcholine on the frog rectus, the rabbit auricle, and the rabbit intestine. It is suggested that the fall of temperature may be due to lessened activity and lessened heat production in the skeletal muscles of the mice.
- 5 A large dose of pethidine causes a rise of temperature accompanied by increased motor activity

I am deeply indebted to Prof J H Burn for suggesting this problem and for the guidance and encouragement he has given in all the stages of this work

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THE INFLUENCE OF POTASSIUM AND CALCIUM IONS ON THE ACTION OF PROCAINE

BY

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It has long been established that the optimum functioning of cells is dependent on optimum amounts of different electrolytes in the fluid surrounding them. In particular it is known that function is still maintained if an excess of one electrolyte is compensated by a corresponding increase of another, the best-known pair being the potassium and calcium ions, chiefly studied in the heart by Ringer

It is important, however, to realize that pharmacological investigations which consider the action of drugs in relation to this variable content of electrolytes are very different from observations of body functions only Electrolytes may prevent or assist the action of a drug on a tissue, and it is not only the affinity of an electrolyte to a cell which is important, but also its affinity to the drug. In the complicated system composed of the cell, a variable content of electrolytes and the drug, with all their mutual relations, the response of the tissue will not necessarily follow the same laws as it would in the absence of the drug

Two recent papers show that the absolute concentration of potassium is of great importance for some drug actions, more so than its relative concentration to calcium, i.e., the K/Ca ratio Baker (1947) tested the interrelation between Ca, K and ouabain on the isolated heart and in the whole animal, Goffart and Brown (1947) investigated the correlation of K and adrenaline on the isolated striated muscle. In order to see whether the effects described are peculiar to these two drugs or have a more general significance. I have tested the effect of changing the K and Ca concentrations on another drug action, namely that of procaine on the refractory period of the isolated rabbit auricles.

METHOD

The method of measuring the activity of pharmacological agents on the refractory period has been described in detail by Dawes (1946a) The auricles,

carefully dissected from the heart of a rabbit, are suspended in a bath containing 100 c c of oxygenated Ringer-Locke at 29° C The composition of the Ringer-Locke is as follows NaCl 9 0 g , KCl 0 42 g , CaCl₂ 0 24 g , NaHCO₃ 0 5 g , dextrose 2 0 g , distilled water 1.000 c c

The contractions of the auricles are recorded on a smoked drum. The auricles contract rhythmically by themselves and they can also be driven by breakshocks from an induction coil at any desired speed. The maximal rate is observed at which the auricles will follow electrical stimulation. The reduction or the increase in maximum rate caused by a drug is calculated and expressed as a percentage of the maximum rate before the drug was applied. This percentage is, within wide limits, independent of the initial maximum rate, as Dawes observed

RESULTS

Procaine

Procaine reduces the maximum rate, ie, it prolongs the refractory period of the isolated rabbit auricles (for references see Dawes, 1946a and b)

The activity of procaine was tested on 9 preparations The effect was measured after the procaine had been allowed to act for a fixed time. The average results of all observations are shown in Table I

TABLE I

Concentration of procaine acting for 10 minutes	3 × 10 ⁻⁶	10-5	2 × 10 ⁻⁸
Percentage reduc- tion of the maxi- mal rate	13 8 ± 0 4	23 9 ± 0 98	34 4 ± 0 88

The effects of procaine were clear, easy to estimate, and the muscle recovered in 25–30 minutes. This relatively rapid recovery was one of the reasons why procaine was chosen rather than any of the other substances with "quinidine-like properties" tested by Dawes (1946a and b)

The spontaneous rhythm was slightly increased, or remained unaltered, by a low concentration of procaine (3 \times 10⁻⁴), while it was decreased by a higher concentration (2 \times 10⁻³), this effect was more pronounced if the original pulse-rate was high

Potassium and calcium ions

Potassium is reported to be without effect on the refractory period of the frog ventricle (Kanda, 1939), although many authors have found that it lengthens the refractory phase (Boehm, 1914, Cicardo and Marenzi, 1938, Lueken and Schuetz, 1939) Calcium has been found to be without any clear effect (Cicardo and Marenzi, 1938)

In my experiments an increase in potassium lengthened the refractory period, for example, the maximum rate was reduced by 23 per cent and the spontaneous rate by 25 per cent, if the potassium concentration of the Locke solution was raised by 50 per cent.

If the auricle was left beating in potassium-free Ringer solution for about 2 hours this had no effect on the refractory period, there was, however, an effect on the pacemaker, since the spontaneous rhythm increased by 40 per cent

When the amount of CaCl, was doubled or tripled, the maximal rate increased by no more

than 3-7 per cent, and even very high concentrations (8 times normal) either did not affect the refractory period or lengthened it by about 5 per cent only, at a time when the spontaneous rate was increased by 47 per cent.

Reduction of the calcium concentration by 50

Reduction of the calcium concentration by 50 per cent prolonged the refractory period, the maximum rate at which the auricle responded to electrical stimuli was reduced by 15 per cent. This decrease resembled the effect on the refractory period produced by increasing the potassium content, but while the pacemaker was depressed by excess K, it was not affected by lowering the calcium concentration to 50 per cent

Effect of K and Ca on the action of procaine

When the influence of changes in K or Ca content on the action of procaine was tested, the change of electrolyte concentration was first allowed to establish its own intrinsic effect for a period of 1 to 3 hours before the procaine was added, this was done in order to ensure that the result observed was not due to summation of the effects of the electrolyte and of procaine Increasing the amount of potassium (Fig 1A) or decreasing the amount of calcium (Fig 1B) both potentiated the action of procaine About the same proportional change was obtained by raising the

K by 50 per cent or by lowering the Ca by 50 per cent.

A reduction of the potassium or an increase of the calcium content produced the same effect qualitatively but not quantitatively, in these conditions the action of procaine was depressed However, in order to obtain the same degree of depression as that produced by reducing the K concentration by 50 per cent, it was necessary to increase the Ca concentration by 600 per cent (see Fig 1C and D), for example, the effect of 2 × 10-3 procaine in Locke solution containing half the normal amount of K was depressed by 66 per cent, but a depression of 64 per cent was only produced by raising the Ca concentration to 7 times the normal amount In another experiment even 8 times more Ca was required

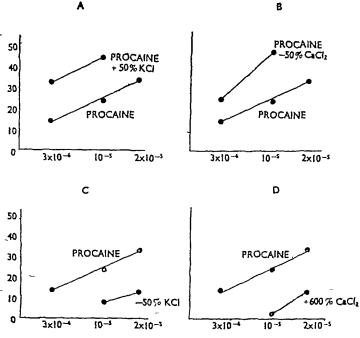


Fig. 1—Effect of procume on isolated rabbit auricles with different K and Ca concentrations. Ordinates percentage reduction in the maximal rate at which the auricles follow electrical stimulation. Abscissae concentration of procume

DISCUSSION

The experiments described show that the influence of potassium and calcium on the refractory period

of the isolated rabbit auricles is antagonistic Although an excess of calcium or a reduction of potassium is without intrinsic effect over a period of 2-3 hours, this change of electrolytes nevertheless influences the action of procaine, demonstrating that an optimal amount of all electrolytes is necessary for the usual response of the cell to drugs, even though an intrinsic effect of variation in the contents of electrolytes itself is not immediately visible The quantitative aspect, however, shows the importance of the absolute concentration of potassium for drug action. Baker (1947) has shown it for the action of ouabain, and in this paper it has been demonstrated for procaine Under the experimental conditions of this investigation the alteration of procaine activity by a reduction of potassium cannot be reproduced by a correspondingly increased amount of calcium, on the other hand the effect of an increased amount of potassium can be imitated by a corresponding loss of calcium, which shows that at least the normal amount of Ca should be present

SUMMARY

(1) The action of procaine on the refractory period of the isolated rabbit auricle was deter-

mined in the presence of variable amounts of potassium and calcium

- (2) Qualitatively, an excess of potassium and a reduction of calcium potentiate the activity of procaine, a reduction of potassium and an excess of calcium diminish the effect of procaine on the refractory period
- (3) Quantitatively, the determining factor is not the ratio K/Ca but the absolute amount of potassium present, although a certain amount of Ca is necessary

I should like to express my sincere thanks to Prof J H Burn for his hospitality and the help given while carrying out this work

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THE CHANGE IN PHARMACOLOGICAL ACTION PRODUCED BY THE INTRODUCTION OF A METHYL GROUP INTO PRISCOL

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Within the last decade a new series of chemical compounds, the 2-substituted imidazolines, have claimed wide attention because of their vascular actions. Several of them, phedracin, priscol, privine and otrivin, have been used clinically in peripheral vascular disorders.

Hartmann and Isler (1939) made a preliminary pharmacological investigation of a large number of these imidazolines They found that small changes in the imidazoline molecule sometimes caused great quantitative or even qualitative changes in the effect upon the blood pressure Similar abrupt transitions have been described among sympathomimetic amines (Beyer, 1946) Since the substitution of an N-methyl group in the noradrenaline molecule causes significant differ ences in its properties (Barger and Dale, 1910) it was decided to investigate what changes the introduction of an N-methyl group would effect in The structural formulae of these compounds are as follows

2-Benzyl-1-methyl-imidazoline was one of the compounds investigated by Hartmann and Isler They stated that it had approximately the same toxicity and effect on the rabbits intestine as

priscol, but caused a rise of blood pressure, whereas priscol caused a fall

The general pharmacology of priscol and its N-methyl derivative is here described as part of an attempt to elucidate the mechanism of action of the imidazolines

EXPERIMENTAL RESULTS

Cardiovascular action of priscol—The action of priscol on the blood pressure varies in different species. Meier and Müller (1939) observed a fall of blood pressure in rabbits, Hermann, Jourdan and Bonnet (1941) found that in dogs priscol usually caused a fall but occasionally a rise of blood pressure. In cats Chess and Yonkman (1945) observed either no effect or a fall with larger doses

Since priscol is structurally related to histamine it seemed possible that the fall of blood pressure might be antagonized by neoantergan However, in a cat under ether anaesthesia, a dose of neoantergan which virtually abolishes the depressor effect of histamine did not prevent the fall of blood pressure caused by a large dose of priscol All the workers quoted above observed that priscol reduced or reversed the pressor action of adrenaline An example of this in the spinal cat is shown in Fig 1 After the injection of 10 mg priscol, which was itself without action on the blood pressure or spleen volume, 10 µg adrenaline caused a small fall of blood pressure accompanied by dilatation of the spleen, the pressor effect and the contraction of the spleen caused by 5 μg adrenaline before the injection of priscol were absent When a larger dose of adrenaline was injected, however, the original effect was seen



Fig 1 —Upper record, spleen volume, lower record blood pressure of spinal cat $\,$ At A, 5 μg adrenaline caused contraction of spleen and rise of pressure At B, 10 mg priscol $\,$ At C, 10 μg adrenaline caused slight dilatation of spleen and fall of blood pressure At D, 01 mg adrenaline caused contraction of spleen and a small rise of pressure

once more, 01 mg adrenaline caused of blood pressure and contraction of the

This observation suggested that th priscol on the pressor response of might be attributed to competition between priscol and adrenaline molecules for the same "receptor" The effect of different amounts of priscol on a range of doses of adrenaline was therefore determined in a series of spinal cats. The results are shown in Fig 2 in which curve A shows the mean height of the pressor response to a given dose of adrenaline in the absence of priscol shows the effect on the adrenaline response of administering priscol in a dose of 25 mg/kg, and curve C that of 5 mg/kg It can be seen from Fig 2 that the reversal of the pressor action of adrenaline by priscol is only possible when small doses of adrenaline are injected, and that the reversal is easily overcome by larger doses

Priscol on blood vessels—The action of priscol on the blood vessels was determined by perfusing the rabbit ear, using the method of Gaddum and Kwiatkowski (1938) In Fig 3 the vasodilatation produced by 0.5 mg priscol is shown. The action on the adrenaline response was also examined in this preparation and the record in Fig 4 shows the reversal of the constrictor action when priscol was added to the Ringer's solution perfusing the ear (a) shows two vasoconstrictor effects due to the injection of 0.1 μ g adrenaline, at (b) in the presence of priscol, 0.8 μ g adrenaline caused

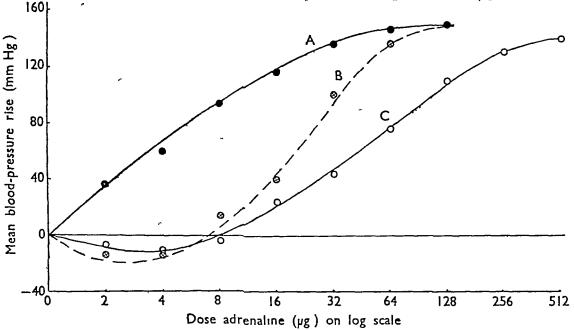
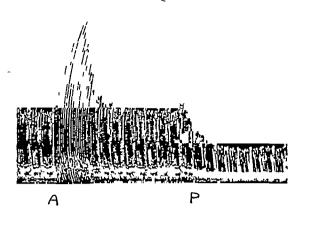


Fig 2—Priscol-adrenaline antagonism on blood pressure of spinal cat Mean blood-pressure rise (ordinate mm Hg) plotted against dose (μ g) adrenaline as abscissa on logarithmic scale Curve A shows blood-pressure rise before priscol (mean of 3 exp), curve B after 2.5 mg/kg. priscol (mean of 2 exp), curve C after 5 0 mg/kg priscol (mean of 3 exp)



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Fig 3—Record of outflow from vessels of rabbit ear obtained by Gaddum's drop timer At A, 0 025 µg. adrenaline caused vasoconstriction, at P, 0 5 mg priscol caused vasodilatation

vasodilatation, and 16 μ g was without effect, at (c) 3-2 μ g adrenaline caused a slight vasoconstriction, and at (d) 4 μ g a constriction similar to the initial effects. Thus not only does priscol cause vasodilatation and reverse the vasoconstrictor action of adrenaline in isolated perfused vessels,

but as in the spinal cat the relationship between priscol and adrenaline is quantitative and not qualitative. Histamine also causes vasoconstriction in the rabbit's ear, larger doses of priscol than those necessary to abolish the action of adrenaline abolished the vasoconstriction caused by histamine, but no reversal was seen.

Similar observations were made when the hindleg of a dog was perfused with blood (containing heparin) by a Dale-Schuster (1928) pump. The injection of 10 mg priscol caused vasoconstriction (in contrast to the vasodilatation seen in the rabbit's ear) and converted the previous constrictor action of 4 μg adrenaline to a dilator action. When 20 mg priscol was injected the dilator action of 4 μg adrenaline became greater and resembled that of 20 μg histamine. The effect of histamine itself was unchanged by priscol

Priscol on the heart — The action of priscol on the heart was examined by perfusing the isolated heart with Locke's solution by Langendorff's method. In two rabbit hearts, the injection of $10-20~\mu g$ priscol decreased the amplitude and reduced the coronary flow. A similar action was observed in three experiments on cat hearts, but in other experiments on cat hearts an increase in the heart's action was observed especially with larger doses (1-3 mg). An example of this is

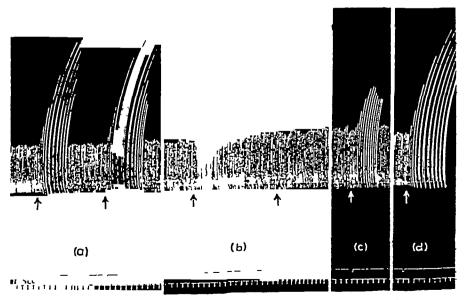


FIG 4—Record as in Fig. 3 In (a) two injections of 0.1 μg adrenaline causing vasoconstriction Between (a) and (b) perfusion with Ringer containing priscol (0.2 mg per ml) was begun In (b) at first arrow 0.8 μg. adrenaline caused vasodilatation, and at the second, 1.6 μg was without effect. In (c) 3.2 μg caused vasoconstriction, and in (d) 4.0 μg. caused an effect similar to that in (a)

given in Fig 5 which shows the effect of 2 mg, this dose not only increased the amplitude but also both the rate (from 102-200 per min) and the coronary flow (from 60-104 ml per 'min.) Although the increase in amplitude did not last more than 8 min the increase in rate and in coronary flow continued for 15 min

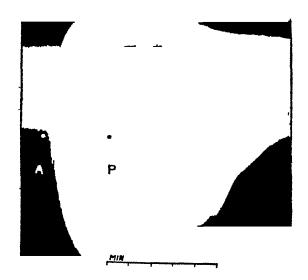
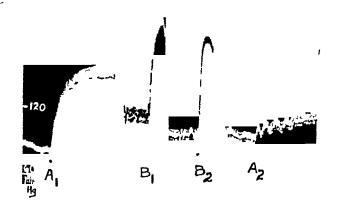


Fig 5—Record of contractions of isolated cat heart perfused by method of Langendorff At A, 0 3 μ g. adrenaline increased force of contraction, heart rate from 104/min to 140/min, and coronary flow from 56 ml/min to 78 ml/min At P, 2 mg priscol increased force of contraction, heart rate from 102/min to 200/min, and coronary flow from 60 ml/min to 104 ml/min. There is prolonged stimulation due to priscol 8 min after injection, heart rate still 173/min and coronary flow 96 ml/

On isolated rabbit auricles at 29° C priscol in concentrations of 2 μ g =4 μ g per ml augmented the amplitude though it slowed the rate Priscol lengthened the refractory period when examined by Dawes's method (1946a), though the concentrations required were large, 20 µg per ml increased it by 6 per cent, and 01 mg per ml increased it by 22 per cent. Priscol did not affect the action of either adrenaline or acetylcholine on the spontaneous beats of the isolated auricles

Cardiovascular action of N-methylpriscol—The N-methyl derivative of priscol, unlike priscol, always caused a rise of blood pressure, as Hartmann and Isler (1939) had observed An example is given in Fig 6 in which the effect of injecting N-methylpriscol (20 mg) is shown at A₁, the prolonged rise of blood pressure was accompanied by At B₁ 10 μg a great increase in heart rate adrenaline was injected A series of doses of nicotine was then given, until no further pressor

action was observed, a total of 315 mg nicotine acid tartrate was required. The injection of 10 μ g. adrenaline at B₂ then caused about the same rise of blood pressure as at B, but the injection of 20 mg N-methylpriscol at A2 was almost without effect. This suggested that the pressor action of N-methylpriscol was due to liberation of adrenaline and it was found that the pressor action of N-methylpriscol was greatly reduced by adrenalectomy



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Fig 6—Record of blood pressure of spinal cat At A₁, 20 mg N-methylpriscol caused rise of blood pressure, at B₁, 10 µg adrenaline had pressor action. Between B₁ and B₂ nicotine was injected until it no longer caused a rise (total injected 31 5 mg) B_2 , $10 \,\mu g$ adrenaline had a similar effect to that at B_1 , but 20 mg N-methylpriscol at A_2 had almost no action on blood pressure

Since the pressor action of N-methylpriscol is largely due to the release of adrenaline, and since priscol abolishes the pressor action of adrenaline, it would be expected that priscol would modify the pressor action of N-methylpriscol proved to be so In Fig 7 a record of the nictitating membrane and of the blood pressure of a spinal cat is shown At A, 20 μ g adrenaline was injected and, at B, 5 mg N-methylpriscol latter caused a small prolonged contraction of the At C, 5 mg/kg priscol nictitating membrane was injected, and the nictitating membrane contracted strongly and persistently At D, the same dose of adrenaline as was previously given at A caused a fall of blood pressure The injection of 5 mg N-methylpriscol at E now had a much smaller pressor action.

N-methylpriscol on the blood vessels—When examined on the vessels of the rabbit's ear, doses of N-methylpriscol up to 1 mg were without action

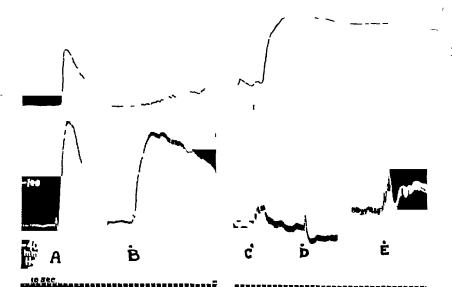


Fig 7 —Upper record contractions of nictitating membrane, lower record blood pressure of spinal At A, 20 µg adrenaline caused contraction of nictitating membrane and rise of blood pressure, at B, 5 mg N-methylpriscol caused slow contraction of nictitating membrane and rise of pressure, at C, 5 mg/kg. priscol caused powerful prolonged contraction of nictitating membrane, now at D, 20 µg. adrenaline was depressor, and 5 mg N-methylpriscol at E had little pressor action

N-methylpriscol on the heart—On the cat heart perfused with Locke's solution through the coronary vessels, N-methylpriscol was found to have a stimulant action as shown in Fig 8 At A, 0.5 mg N-methylpriscol increased the rate from 120 to 206 per min, the coronary flow from 9.0 to 15.0 ml per min. and the amplitude as shown in the figure At B, 50 µg nicotine produced a similar effect but of much shorter duration Since N-methylpriscol caused a rise of blood pressure

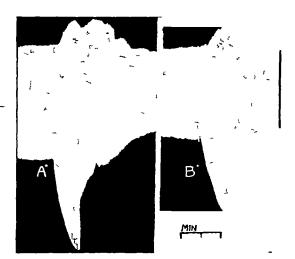


Fig 8—Record of contractions of isolated cat heart perfused by method of Langendorff At A, 0.5 mg N-methylpriscol increased force of contraction, heart rate from 120/min to 206/min, and coronary flow from 9.0 ml/min to 15.0 ml/min At B, 50 µg nicotine acid tartrate increased amplitude of contraction, heart rate from 128/min to 160/min, and coronary flow from 8.6 ml/min to 11.0 ml/min

and increase of heart rate by a "nicotine-like" liberation of adrenaline from the adrenals, it was expected that its action upon the isolated-heart would be another manifestation of the same "nicotine-like" property. But it was found that perfusion of the cat's heart with nicotine (2 mg per ml.) abolished the stimulant action of nicotine, but not that of N-methylpriscol. In this respect N-methylpriscol resembles adrenaline and hista mine, rather than nicotine, in its action upon the isolated heart.

N-methylpriscol increased the rate and the amplitude of contraction of the isolated rabbit auricles, again it differed from nicotine, since tetraethylammonium iodide abolished the action of nicotine, but had no effect on that of N-methyl priscol, nor was the action of N-methylpriscol reduced by the antihistamine substance neoantergan.

N-methylpriscol did not affect the refractory period of the electrically-driven auricles even in a concentration as high as 10⁻⁴, though this was enough to increase the spontaneous rate and the amplitude

Relation to amidines—The chemical structure of the imidazoline ring is related to that of the amidines. Dawes (1946b) showed that the latter potentiated the pressor action of adrenaline when injected into the splenic vein so as to pass through the portal system before entering the general circulation. Similar experiments were therefore performed with N-methylpriscol. Fig. 9 shows the blood pressure responses of a spinal cat to six injections of 20 µg adrenaline, 5 mg. N-methylpriscol was mixed with the adrenaline in the

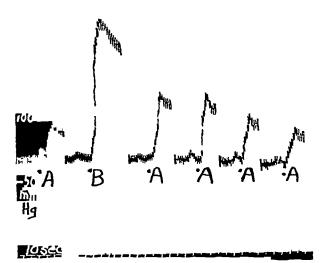


Fig 9—Record of blood pressure of spinal cat, all injections made into portal circulation through splenic vein. At each A, 20 µg adrenaline produced rise of blood pressure, at B, 5 mg N-methylpriscol was mixed with 20 µg adrenaline and caused much greater rise of blood pressure.

second injection The effect of the second injection was much greater than that of the first, and the succeeding injections progressively declined Since the injection of 5 mg N-methylpriscol alone had no effect on the blood pressure when given

by this route, it clearly potentiated the action of adrenaline

Smooth muscle—The action of priscol was studied in the ánaesthetised cat by inserting balloons in the oesophagus, duodenum and colon. In some experiments the vagi were stimulated below the heart, and in others the spontaneous intestinal movements were increased by the injection of eserine. Fig. 10 shows the record of the duodenal contractions, they were inhibited by adrenaline, a large contraction was induced by priscol (5 mg/kg), 13 min. later another injection of adrenaline increased the contractions of the intestine, at the same time causing a fall of blood pressure. This reversal of the effect of adrenaline on the duodenum was not seen in other parts of the gastro-intestinal tract.

In the isolated duodenum of the rabbit priscol reduced the inhibitory action of both adrenaline and privine, but did not reverse their action

Both priscol and N-methylpriscol were found to potentiate the action of acetylcholine on the guinea-pig ileum, while N-methylpriscol also potentiated the action of histamine in the guinea-pig ileum as shown in Fig 11. It should, however, be observed that the spontaneous movements were also increased

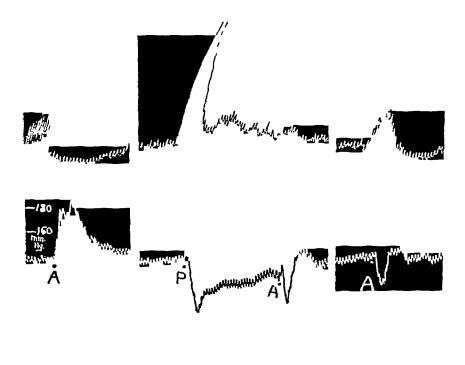


Fig 10—Upper record contractions of duodenum recorded by intestinal balloon, water manometer and piston recorder, lower record blood pressure of cat under chloralose anaesthesia At the first A, 10 µg adrenaline inhibited duodenum and caused rise of blood pressure, at P, 5 mg/kg priscol caused fall of blood pressure and large contraction of duodenum, 3 min after priscol 10 µg adrenaline, at second A, depressor action but no effect on duodenum, 13 min after priscol 10 µg adrenalme, at last A, caused fall of blood pressure and contraction of duodenum

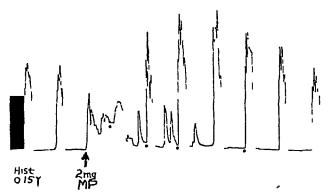


Fig 11—Record of contractions of isolated guinea-pig ileum in Tyrode solution All contractions caused by 0.15 µg. histamine except at arrow where 2 mg N-methylpriscol was added to bath and left in for 30 sec before the next 0.15 µg histamine was added (at dot). Note the increased spontaneous activity after N-methylpriscol

Skeletal muscle—Priscol and N-methylpriscol potentiate the contractions of the frog's rectus abdominis caused by acetylcholine—When the cat's sciatic nerve is stimulated by maximal single shocks (16 per min) the injection of either priscol or N-methylpriscol into the central end of the external iliac artery near the bifurcation caused a curariform depression of the contractions of the gastrocnemius muscle as shown in Fig 12—The same effect was observed in the phrenic nerve diaphragm preparation of the rat (Bulbring, 1946), and in the frog gastrocnemius

Superior cervical ganglion —When the superior cervical ganglion of the cat was perfused with Locke's solution, and the contractions of the nictitating membrane were recorded, it was found that

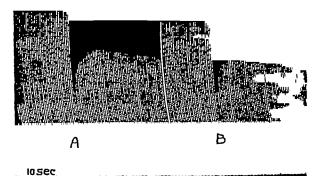


Fig 12—Record of contractions of gastrocnemius muscle of spinal cat caused by supra-maximal electrical stimulation (rate 16/min) of sciatic nerve. At A, 8 mg N-methylpriscol injected into external iliac artery had curariform action, and at B, 8 mg priscol had same action

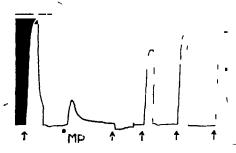


FIG 13—Record of contractions of nictitating membrane caused by supra maximal electrical stimulation of pre-ganglionic fibres of cat's superior cervical ganglion perfused with warm Locke solution At arrows stimulations lasting 15 sec (given every 3 min) At MP, 0.5 mg N-methylpriscol caused contraction, it abolished the contraction due to the next stimulation, and reduced the second succeeding contraction

both priscol and N-methylpriscol when injected into the perfusing fluid caused a contraction of the membrane. The contraction due to N-methyl priscol was more rapid in onset and in relaxation than that due to priscol, in contrast to the effect produced in the whole animal (Fig. 7). The injection of N-methylpriscol also abolished the effect of the next stimulation of the preganglionic fibres as shown in Fig. 13.

Gastric secretion —When priscol was infused at a uniform rate into a vein it was found to cause a large output of gastric juice (collected by a wide cannula introduced through the pylorus in the stomach), this was observed in six cats anaesthe tised with pentobarbitone When N-methylpriscol was infused in four times the concentration, it failed to produce any secretion in two cats

DISCUSSION--

The observations described in this paper show that the reversal of the constrictor action of adrenaline by priscol, which is seen in the whole animal, can also be observed in the perfused vessels of the rabbit's ear. It is interesting to record that Rothlin (1925) found that ergotamine also would reverse the action of adrenaline in isolated vessels. The reversal of the pressor action of adrenaline is usually demonstrated in the whole animal, the fact that it also occurs in isolated vessels makes it more probable that it is due to a direct action of adrenaline itself, rather than to liberation of, for instance, histamine, as has been suggested by Staub (1946)

The observations here described show further that the introduction of a methyl group into the imidazoline ring of priscol changes the vascular action so that the peripheral vasodilatation is no longer seen, instead a pressor action appears which can be attributed to a release of adrenaline by stimulation of the sympathetic ganglia pressor effect of N-methylpriscol is greatly reduced by the administration of either full doses of nicotine or of tetraethylammonium iodide, and also by the removal of the suprarenal glands It is therefore very curious that though N-methylpriscol stimulates the isolated cat heart and the rabbit auricles, this does not appear to be a nicotine-like action, but may more properly be compared to the effect of adrenaline In cardiac tissue the action of N-methylpriscol was in some respects similar to that of priscol

While priscol converts the motor effects of adrenaline on the vessels to inhibitor effects, it reduces the inhibitory effects of adrenaline on the intestine, and in at least one instance converts them to motor Thus after the injection of priscol which itself caused a contraction of the cat's duodenum (in vivo), the injection of adrenaline caused a contraction The observations are very similar to those which have been made with ergotoxine and ergotamine, for these substances also convert the motor action of adrenaline on the vessels to an inhibitor action and as Planelles (1925) found, convert the inhibitor action on the intestine to a motor action Priscol potentiated the action of acetylcholine on the intestine

On skeletal muscle priscol and N-methylpriscol were alike in action. On the one hand, the stimulation of the frog rectus by acetylcholine was augmented, and on the other the contractions of the cat's gastrocnemius, when evoked by stimulation of the sciatic nerve, were diminished as by an injection of tubocurarine This curariform action was also observed in the phrenic-diaphragm preparation of the rat In the perfused superior cervical ganglion there was again evidence of a double action Both priscol and N-methylpriscol, on injection into the perfusion fluid, stimulated the ganglion, after the injection of N-methylpriscol, stimulation of the preganglionic fibres was temporarily ineffective We therefore have in priscol and in N-methylpriscol substances which augment or imitate the action of acetylcholine in the sympathetic ganglion and in skeletal muscle, in certain circumstances they have, as nicotine has, the opposite, curariform, action as well.

Perhaps the most striking conclusion to which we are driven by a study of the pharmacology of these substances is that they will not fit into any of the provisional classifications of drugs which in the past have proved so useful. This point can be more effectively illustrated by the following table —

	Priscol	N-Methylpriscol		
Blood pressure Adrenaline action	Fall	Nicotine-like rise		
on blood pressure	Reversed	Unaffected		
Adrenaline pres- sor effect after	~			
portal injection	?Unaffected	Augmented		
Blood vessels	Vasodilatation	No direct effect		
Adrenaline effect	•			
on vessels	Reversed	Unaffected		
Smooth muscle	Abolishes adre-	No effect on response to adrena-		
	Reverses adren- aline in vivo (cat)	line		
Spleen in vivo	Slight dilatation	Contraction		
Cardiac muscle	Stimulated	Stimulated		
Refractory period	Prolonged	Unaffected		
Nictitating mem- brane	Strongly stimu- lated (ganglion removed)	Slight stimulation		
Gastric secretion	Stimulated	Unaffected		
Skeletal muscle	Like nicotine	Like nicotine		
Superior cervical ganglion	Like nicotine	Like nicotine		

It will be impossible to explain their mode of action until we have achieved more knowledge of the physiology of these tissues, but two points may provide a lead for subsequent investigations. The first is the structural relationship between the imidazolines, adrenaline and histamine, and the second is the observation that the reversal of the pressor action of adrenaline by priscol is dependent upon the relative concentrations of the two substances

SUMMARY

- I Reversal of the constrictor action of adrenaline by priscol, demonstrated in the whole animal, can also be observed in the perfused vessels of the rabbit's ear. The dilator action of adrenaline in these circumstances is more likely to be due to the action of adrenaline itself, rather than to the liberation of a substance such as histamine
- 2 The introduction of an N-methyl group into priscol changes the vascular action so that, instead of peripheral vasodilatation, a pressor action is observed, which can be attributed to a release of adrenaline by stimulation of the sympathetic ganglia. The stimulating effect of N-methylpriscol on the isolated heart, however, does not appear to

be a nicotine-like action, but may be compared to the effect of adrenaline In cardiac tissue the action of priscol is, in some respects, similar to that of N-methyl derivative

- 3 Priscol reduces the inhibitory effects of adrenaline on the intestine, and in at least one instance converts them to motor. Both priscol and N-methylpriscol potentiate the action of acetylcholine on the intestine.
- 4 In the sympathetic ganglion and in skeletal muscle priscol and N-methylpriscol augment or imitate the actions of acetylcholine, and in certain circumstances they have the opposite, curariform, action as well
- 5 A study of the pharmacology of these substances indicates that they cannot be fitted into the provisional classifications of drugs which have hitherto been so valuable

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ANALYSIS OF ANTIMITOTIC ACTION OF CERTAIN QUINONES

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The study of mitotic inhibition in experiments dealing with toxicological and pharmacological problems has been used by Dustin (1934) with great success He worked with normal tissues and tested antimitotic activities in vivo The very valuable method of tissue cultures has been applied by Ludford (1936) for the same purpose Other biological materials for the investigation of physiological processes on the same lines have been adopted by Tondury (1941), Lehmann (1942) and Luscher (1946) Tondury worked with Triton eggs. Lehmann (1945, 1946) and later Huber (1947) with the eggs of Tubifex, Lüscher investigated regeneration in Xenopus larvae under the influence of colchicine Barber and Callan (1943) studied the effect of cold and colchicine in mitoses of the

From the beginning it was clear that the study of mitotic inhibitors may well pave the way for a chemotherapeutic approach to the cancer prob-The great variety of antilems (Dustin, 1939) mitotic substances and the lack of any chemical connexion between many of them seemed to be Furthermore nearly all the a serious obstacle potent antimitotics are poisonous to normal cells at concentrations at which they exert antimitotic action on tumour cells, in this way restricting the use of antimitotics to external application on surface tumours Broderson (1943) has given evidence that it is possible by this method to control the growth of malignant tumours of the skin, he used colchicine, whose strong activity as a mitotic inhibitor was well known

Nevertheless these difficulties might be overcome Mitchell (1942) and v Euler and Hevesy (1942) have shown that therapeutic doses of xand gamma-radiations inhibit the synthesis of thymonucleic acid in proliferating normal and malignant cells Substances able to interfere with the metabolism of nucleoproteins ought therefore to be endowed with antimitotic properties, and conversely some mitotic poisons should be able to interfere with the metabolism of nucleoproteins, thus the mechanism by which disturbances in the metabolism of nucleoproteins are induced becomes a possible common denominator for the various antimitotic substances even when they are chemically unrelated To give an example according to Kopac (1945) stilbamidine blocks mitosis in some neoplastic cells, it dissociates protamineribonucleates, releasing the protamine and simultaneously trapping the ribonucleate complex by the formation of an insoluble stilbamidine-ribonu-Nucleoproteins, therefore, can be mactivated by low concentrations of stilbamidine Lettré and his collaborators (1946) found that substances of the type R-Hg-X, where R is an alkyl or an aryl residue, X an inorganic or organic anion or a phenol, are mitotic poisons. They explain the antimitotic activity of these compounds by pointing out that nucleic acids and nucleoproteins are able to combine by salt or complex formation with the organometallic compounds to give insoluble The similarity in the mechanism by which stilbamidine and the organometallic compounds disclose their antimitotic properties is easily seen They belong to the same physiological group of antimitotics, although they are chemically as dissimilar as possible

As mentioned above, the second great difficulty for the therapeutic utilization of antimitotics is the fact that the concentration at which they display the antimitotic activity is nearly always within the range of their toxicity for normal cells. Two ways of overcoming this difficulty have been investigated. The molecule may be changed chemically until it has the required properties, either by investigating the corresponding homologous series or by introducing new radicals in the hope of improving the therapeutic index. The other way is to

cover the active group by substitution The work of the cell consists then in releasing the active principle within the cell This is possible if the cell contains enzymes capable of splitting off the substituent The choice of the substituent is therefore of primary importance. In view of the role which phosphoric acid plays in the synthesis of nucleic acid and the ease of its transfer in the processes supplying the necessary energy for these and other reactions, the phosphate residue stands out as the most promising substituent. In 1946 Mitchell started to study the therapeutic effects of the diphosphate of 2-methyl-1 4-naphthohydroguinone, in combination with radiation, in cancer patients and later gave evidence in collaboration with Simon-Reuss (1947) that this diphosphate produces mitotic inhibition in chick fibroblasts and in some human carcinomata, with potentiation of the mitotic inhibitory effects of x-radiation under suitable conditions in the tissue cultures The mitotic inhibition produced by the unsubstituted 1 4-naphthohydroguinone was discovered by Lehmann (1942), who found it active at a concentration of 10-7, the same degree of activity was displayed by the corresponding quinone Lehmann worked with Tubifex eggs Meier and Allgower (1945) and Meier and Schar (1947) obtained similar results with chick fibroblast cultures

In our own experiments we followed two lines of research. We tried to ascertain the chemical group in the quinone molecule, common to the antimitotics under investigation and responsible for their chemotherapeutic activity. We tried further to get some evidence which would allow us to correlate our results with physiological processes, the disturbance of which may cause interruption of growth, as shown by mitotic inhibition

The present communication is the first of a series of papers dealing with these problems

METHODS

All the experiments were carried out on tissue cultures of chick fibroblasts The tissues used were taken from the sclerotic and choroid of a 10-11-days-old chick embryo The medium consisted of equal parts of fowl plasma and of 15 per cent embryo extract. The usual hanging drop technique was applied Two groups of six cultures of the 4th passage were selected and matched for experimental purposes and controls The compound to be tested was dissolved in Tyrode solution and the pH adjusted to 74 was suitably diluted with Tyrode and sterilized by filtration through Seitz filter Sterilization by boiling in Tyrode was avoided because the boiling of maleic and fumaric acids in Tyrode solution gave unreproduceable results The diluted solution of the substance was then added to the embryonic extract. The final

molar concentrations appear in Table I The cultures were fixed and stained with Heidenhain's haematoxy lin at given intervals and mutotic counts were carried out

EXPERIMENTAL

Many chemical properties of the quinones are centred in the non-benzenoid part of the molecule and the same may apply to the physiological activities. Thus the question arises whether the benzene ring of the naphthoquinones (V) is necessary for the mitotic inhibition induced by them. Maleic acid (I), its methyl derivative, citraconic acid (II), and their trans-isomers, fumaric acid (III), and mesaconic acid (IV), were therefore investigated

I Maleic acid

Pure maleic acid was prepared by hydrolysis of maleic anhydride. The results obtained with maleic acid are given in Table I and show that maleic acid has strong antimitotic properties. At a concentration of 0.5 × 10⁻⁸ molar the inhibition was 33 6 and 54 2 per cent in two experiments The mitotic inhibition increased with rising concentration The increase follows roughly a Togarithmic graph, when plotted against the log 10 of the concentration (Fig 1) The phase distribu tion calculated in percentage of the total mitoses gives no decisive information Microscopically the picture common to all substances with antimitotic activity was observed clumped metaphases and undivided telophases were seen in all phases, frag mentation of chromosomes occurred in all phases, but mainly in anaphases At higher concentrations

TABLE I

TISSUE CULTURES CHICKEN FIBROBLASTS, HANGING DROP METHOD, 4TH PASSAGE, 24 HOUR CULTURES, FIXED IN SUSA, STAINED IN HEIDENHAIN'S HAEMATOXYLIN

	Malaa	Mitoses as % of mitoses of controls	Per cent inhibition	Phase distribution in % of mitoses					
Exp	Molar conc			Prophase	Metaphase	Anaphase	Telophase		
MALEIC ACID (10,795 mitotic cells investigated)									
1 2 3 4 5 6 7 8 9	$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	$ \begin{array}{c} - \\ 458 \pm 38 \\ 326 \pm 50 \\ 455 \pm 82 \\ - \\ 664 \pm 52 \\ 157 \pm 34 \\ 60 \pm 14 \\ - \\ 355 \pm 22 \\ 324 \pm 68 \\ 234 \pm 31 \end{array} $	54 2 67 4 54 5 33 6 84 3 94 0 64 5 67 6 76 6	11 8 17 8 15 7 12 3 16 8 18 1 24 9 18 6 22 5 19 0 18 9 18 4	38 1 30 4 21 3 46 0 29 5 39 4 25 8 - 31 4 25 6 44 6 47 6 38 5	3 4 3 5 3 8 6 1 4 4 3 0 3 0 3 9 5 9 7 8 7 2 4 5	46 8 51 2 59 2 35 6 49 2 39 6 46 4 46 1 46 0 28 2 26 2 38 5		
		CITRACONIC A	ACID (5 449 n						
3 2	Controls	$ \begin{array}{c} $	— — —	21 1 21 9 13 5 13 4	29 3 33 7 37 5 38 6	7 5_ 4 3 9 5 9 9	42 1 40 0 39 5 38 1		
		FUMARIC A	CID (7,841 mi	totic cells inve	estigated)		_		
4	Controls 1 × 10 ⁻⁴ 3 × 10 ⁻⁵ Controls 3 × 10 ⁻⁶ 2 × 10 ⁻⁶ 1 × 10 ⁻⁶	$ \begin{array}{c} $	 15 2 	27 1 26 9 28 5 19 7 21 9 22 9 21 0	28 7 30 9 22.4 29 5 25 9 36 1 34 6	18 45 47 61 99 77 55	41 8 37 7 44 5 44 7 42 3 33 2 38 9		
	Mesaconic Acid (3,374 mitotic cells investigated)								
	Controls 5 × 10 ⁻⁶ 3 × 10 ⁻⁶ 4 1 × 10 ⁻⁶	100 ± 3 5 100 ± 6 9 100 ± 3 3	<u>-</u> -	18 4 30 4 19 4 16 4	33 9 15 7 19 9 15 4	4 4 3 3 4 9 5 3	43 4 50 7 55 7 62 9		
	Tetrasodium	1 4-Nарнтнонург	ROQUINONE DI	рноѕрнате (5,	838 mitotic ce	lls investigated	i)		
4 8	Controls 1×10^{-6} 5×10^{-7} Controls 3×10^{-8} 1×10^{-9} 5×10^{-9} Controls Controls	21 5 ± 4 8 35 7 ± 5 8 — 8 4 ± 2.5 19 4 ± 3 5 41 0 ± 9 1	78 5 64 3 — 91 6 80 6 59 0	28 0 3 4 5 2 16 9 7 0 10 6 21 9	34 7 86 2 86 2 22,9 69 0 55 9 38 0 23 0	12 07 03 17 14 50 58 2.3	36 0 9 7 8 3 58 8 22.5 28 6 34 2 54 9		
1	$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	49 1 ± 6 1 77 7 ± 8 9 86 2 ± 9 7	50 9 22 3 13 8	20 7 16 9 19 5	24 9 35 8 28 6	4 2 2.7 3 8	50 2 44 7 48 1		
	1 4-Naphthohydroquinone Mono-Hydrogen Succinate (7,557 mitotic cells investigated)								
5	$ \begin{array}{c cccc} & Controls & & & 1 \times 10^{-9} \\ 3 & & 3 \times 10^{-9} \\ 4 & & 5 \times 10^{-9} \\ Controls & & & 1 \times 10^{-6} \\ 6 & & 3 \times 10^{-6} \\ 7 & & & 5 \times 10^{-6} \\ \end{array} $	$ \begin{array}{c} $	— — — — —	20 6 21 7 22.7 22.5 13 5 14 4 14 7 14 0	38 7 30 1 29 0 30 0 36 9 43 9 42.7 44 9	57 47 49 44 39 57 60 46	35 0 43 4 43 6 43 1 45 8 36 0 36 6 36 5		

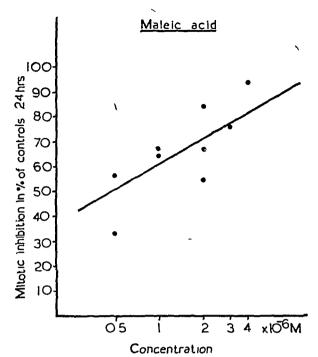


FIG 1—Mitotic inhibition plotted as percentage of controls against the logarithm of the concentration of maleic acid

(3 and $4 \times 10^{-6} M$) the cultures showed but little outgrowth, the resting cells were flattened out and some exploded cells could be seen

II Citraconic acid

The methyl derivative of maleic acid, citraconic acid, showed no mitotic inhibition. There was no difference in the phase distributions compared with the controls (Table I). Microscopically a few clumped metaphases were to be seen but no other abnormalities.

III Fumarıc acıd

Table I shows that fumaric acid induces no mitotic inhibition. In the phase distribution a slight increase only in anaphase is to be noted Microscopically all concentrations showed slight clumped metaphases and mitotic disturbances chromosome breakages in late metaphases and anaphases were seen, only the telophases seemed not to be affected Microscopical pictures were found which resembled those obtained with maleic acid Abnormal cells were present, and the proportion in which they were present was very near that observed in normal tissue cultures, viz., 5 per cent (Found abnormal mitoses in per cent of the mitoses in the experiment at $1 \times 10^{-6}M$ 66 + 07 per cent from 377 cells in mitosis, at

 $2 \times 10^{6} M$ 9.2 ± 1.72 per cent from 338 cells 1.1 mitosis, at $3 \times 10^{-6} M$ 4.6 ± 0.97 per cent from 305 cells in mitosis)

IV Mesaconic ac d

Mesaconic acid (methyl fumaric acid) (Table I) showed no mitotic inhibition and no abnormal cells were found. The phase distribution seemed to indicate an increase in telophase in the lower concentrations which would have to be investigated further.

V I 4-Naphthohydroquinone diphosphate

Lehmann (1942) has already shown that 1 4-naphthoquinone is a stronger antimitotic than its 2-methyl derivative. He worked with *Tub fex eggs*. Meier and Allgower (1945) and Meier and Schar (1947) tested the substance in tissue cultures and described the cytological pictures obtained

As quinones and hydroquinones have apparently the same degree of antimitotic activity (Lehmann, 1942) we prepared the previously unknown 1 4-naphthohydroquinone diphosphate in order to ascertain quantitatively its activitý in tissue cultures

Table I gives the results It'will be seen that 1 4-naphthohydroquinone diphosphate is an extremely powerful inhibitor of mitosis. Compared with the results calculated by Huber (1947) for the antimitotic activity of 1 4-naphthoquinone a remarkable agreement is to be noted. Huber found 1 4-naphthoquinone active at 0.7 × 10 M for Tubifex eggs, whereas our experiments on chick fibroblasts show threshold activity of 1 4-naphthohydroquinone diphosphate at 0.5 × 10 M. Compared with the 2-methyl derivative, tested by Mitchell and Simon-Reuss (1947), the activity of

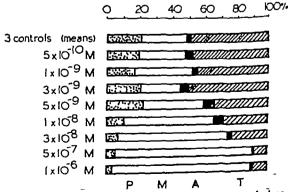


FIG 2—Tetrasodium 1 4-naphthohydroquinone diphosphate Phase distribution as percentage of total mitoses normal from 5 × 10⁻¹⁰ to 3 × 10⁻⁹M, increase of metaphases from 5 × 10⁻⁹M

the new methyl free derivative is more than 1,000 times greater \ With rising concentration the activity of the diphosphate increases From 5×10^{-10} $-1 \times 10^{-8}M$ the increase follows a logarithmic The phase distribution (Fig 2) is nearly normal from $5 \times 10^{-10} M - 3 \times 10^{-9} M$, but at 5 \times 10⁻⁹M the metaphases are already increased This increase grows continuously with rising concentration until at $5 \times 10^{-7} M$ 86 per cent of the total mitoses are in metaphase In the whole range of 10°M, the range of its mitotic inhibition, no toxic effect was observed, although the general picture found in mitotic disturbances was well in evidence clumped metaphases, in all phases some chromosome breakages, some enlarged metaphases and undivided telophases Toxic effects began to appear at 10-8M, vacuolized and exploded cells being found Abnormal cells were investigated for the concentration range $0.5 \times 10^{\circ} - 1 \times$ 10-8M

VI 1 4-Naphthohydroquinone mono-hydrogen succinate

This substance-was prepared in order to see whether the phosphate residue in the 1 4-naphthohydroquinone diphosphate could be replaced by another acidic group

Table I shows that 1 4-naphthohydroquinone mono-hydrogen succinate had no effect on mitosis. The phase distribution seemed to be slightly affected, but the figures allowed no clear interpretation. Microscopical investigation showed the absence of abnormal mitoses.

DISCUSSION

In this paper we have used tissue cultures as a method of approach to the chemotherapy of growth inhibition. Mitotic inhibition, phase distribution, and the cytological picture have all been studied as indices of the effects exerted on tissue growth by the different agents. The picture disclosed by the three parts of this analysis may seem to give a reliable basis for the chemotherapeutic evaluation of the substances tested, this is obviously only true within certain limits, although the results given in the three parts of this analysis have been obtained by quantitative methods

The study of phase distribution alone for instance gives little reliable information. Maleic acid, a strong inhibitor of mitosis, shows no appreciable change in phase distribution (Table I) Furthermore disturbances in phase distribution may precede mitotic inhibition at lower concentrations (unpublished data) or they may follow mitotic inhibition after the peak of the inhibition has

nearly been reached (1 4-naphthohydroquinone diphosphate, Table I, experiment 7), or one set of experiments may display a shift of the phases in one direction, another set in the opposite direction (Table I, 1 4-napthohydroquinone mono-hydrogen succinate)

Mitotic disturbances, as shown by the occurrence of abnormal cells, without mitotic inhibition and without alteration in phase distribution have not been prominent with the substances examined in this paper, but they occur, as shown by v Mollendorff (1938), and the slight indications of such a possibility, encountered in the study of fumaric acid, convey a warning which cannot be dismissed quantitative determination of abnormal mitoses in connection with mitotic inhibition may provide useful chemotherapeutic information. special investigation is planned to elaborate these relations The figures given for mitotic inhibition seem to have a greater individual importance than those for phase distribution They are the result of many mitotic processes and represent an average expression of them Their fluctuations, recorded statistically, are, as a rule, within reasonable limits and allow their use as a quantitative expression of mitotic inhibition Thus the positive statement of mitotic inhibition of a definite degree for a given concentration is fairly safe The negative statement, no mitotic inhibition, based only on the absence of mitotic inhibition, may be erroneous,

Taking mitotic inhibition to express our results the experiments can be summarized as follows

The results show that the ring structure of the quinones is not necessary for inducing antimitotic activity. Maleic acid (1) which contains the aliphatic part of the quinone molecule is a strong antimitotic. In this connection it may be remembered that Brunschwig et al. (1946) were able to show that the growth of cancer in rats can be retarded by maleic acid.

Introducing a methyl group into maleic acid gives citraconic acid (II), which does not show the antimitotic properties of maleic acid

The importance of the cis-configuration of maleic acid is shown by the behaviour of fumaric acid (III) and mesaconic acid (IV), the trans-

isomers of maleic and citraconic acids. Fumaric acid exerts no mitotic inhibition, in agreement with the results obtained by Pomerat and Wilmer (1939), mesaconic acid was also completely inactive.

A methyl group introducted into maleic acid has obviously a dystherapeutic effect. The same is true in the 1 4-naphthoquinone series. The methyl free 1 4-naphthohydroquinone diphosphate belongs to the most powerful antimitotics obtained so far. Compared with colchicine and taking the figures given by Huber (1947) for its antimitotic action on $Tubifex\ eggs$ as basis (8 41 × 10- 3M) it is approximately 15 times more active than colchicine

The importance of phosphorylation, inaugurated in this series by Mitchell and Simon-Reuss (1947) with the examination of the diphosphate of 2-methyl-1 4-naphthohydroquinone, is again apparent Introducing succinic acid instead of phosphoric acid in 1 4-naphthohydroquinone gave 1 4-naphthohydroquinone mono-hydrogen succinate (VI) which was completely inactive. The role of the phosphorylation will be discussed in another paper of this series

The present investigation discloses clearly the unit which maleic acid and the quinones have in common We find in both groups the same uninterrupted system of three conjugated double bonds in which the second double bond is activated by the two adjacent C=0 groups. The experimental evidence, so far obtained, seems to suggest that the unit 0=C-CR=CH-C=0, R=H, in maleic acid and R=H or CH_3 in the quinones, is connected with the antimitotic activity of this group

This unit is closely related to stilbylamine, which Lettré (1946) has shown to be the active principle in the great colchicine group, basing his deduction on the Windaus formula for colchicine, although not disregarding the alternative structure put forward by Cook et al (1944) and by Dewar (1945) It is also related to stilbene, enclosed in the stilboestrols and investigated in tissue cultures by von Mollendorff (1939) and by Lettré and Albrecht (1941 and 1943)

CHEMICAL SECTION

Preparation of 1 4 - naphthohydroduinone diphosphate and its salts Dibarium salt - A stream of nitrogen was passed into a flask containing phosphorous oxychloride (9 c c) in pure dry pyridine (60 c c.) The contents were stirred in ice during the dropwise addition over 45 minutes of an ice-cold solution of 1 4-naphthohydroquinone (3 5 g) in pyridine (90 c.c) After final addition, the mixture was stirred in ice for 30 minutes and then evaporated in vacuo under nitrogen at 50° When the residue was almost dry. pyridine (50 cc) was added and the evaporation re-The residue was left overnight in vacuo over sulphuric acid Water (200 cc) was then added, the whole well shaken and left at room temperature for Hyflo supercel (ca 1 g) was added, the liquid filtered under reduced pressure, and the pale vellow filtrate made just alkaline to phenolphthalein by addition of a saturated solution of barium hydroxide (ca 1 litre) The small precipitate was filtered off after addition of Hyflo and the filtrate evaporated in vacuo under nitrogen at 70° to about 500 c.c. The concentrate was filtered through a layer of Hyflo on a Buchner funnel and the filtrate added slowly with shaking to alcohol (500 cc) The flocculent precipi tate was left for 36 hours at room temperature in the dark after displacing air with nitrogen The product formed a gel which was centrifuged off, washed once with 50 per cent aqueous alcohol, once with alcohol, and finally with ether The residue was dried in vacuo over silica gel Crude yield 13 3 g colourless amorphous solid The salt appears to decompose when heated in solution

Tetracyclohexylamine salt—A solution of cyclohexylamine (5 cc) in water was titrated with N sulphuric acid till a pH of 3 8 was reached (43 5 c.c 1006N sulphuric acid required) This solution was diluted to 100 cc with distilled water

Dibarium 1 4-naphthohydroquinone diphosphate (3.2435 g, crude) was dissolved almost completely in water (100 cc) and titrated with the above cyclohexylamine sulphate solution till only a trace of free barium ions remained (as tested for by an aqueous This required solution of sodium rhodizonate) 445 cc, if the barium salt had been pure, 535 cc. would have been needed Hyflo supercel was added, the precipitated barium sulphate filtered off through a layer of Hyflo and the filtrate evaporated to dryness The gummy residue in vacuo over nitrogen at 60° was dissolved in methyl alcohol (25 cc), decolorized with charcoal, and evaporated to about 10 c.c Ether was added till a slight opalescence appeared standing overnight prisms of the tetracyclohexylamine salt of 1 4-naphthohydroquinone diphosphate separated which were filtered off and washed with methyl alcohol-ether (1 1), then with ether, and dried, 21 g colourless prisms (yield 50 per cent) m p 193 5-194 For analysis, a (decomp after sintering at 192°) specimen was recrystallized from methyl alcohol, m p unchanged (Found C, 546, H, 90, N, 745 per cent. C_{1.8}H_{1.0}O₁P₂ 4C₂H_{1.3}N₂2H₂O requires C, 54.2. H. 88, N. 744 per cent)

Tetrasodium salt —The tetracyclohexylamine salt of 1 4-naphthohydrogumone diphosphate (1 7330 g) in water (25 c c) was treated with the theoretical amount of sodium hydroxide solution (96 5 cc of 0 0956N), the resulting solution filtered through a sintered glass funnel, a little redistilled hexyl alcohol added, and the whole evaporated in vacuo over nitrogen at 70° colourless only residue gradually crystallized when left The material was ground up with alcohol in an agate mortar, filtered off and dried sodium salt of 1 4-naphthohydroquinone diphosphate crystallized in colourless prisms, 08 g (yield 85 per cent) (Found, in material dried at 50° C, 245, H, 32 Loss in weight at 180°, 190 C₁₀H₆O₈P₂Na₄ 5H₂O requires C, 241, H, 32 Loss of 5H₂O requires 181 per cent) The salt is soluble in cold water to form a neutral solution, and forms a gel on addition of organic solvents

1 4-Naphthohydroquinone diphosphate—(a) From the tetrasodium salt. Tetrasodium salt of 1 4-naphthohydroquinone diphosphate (0 45 g) was treated with ca 5N hydrochloric acid (1 1 cc) and water (5 cc) The solution was evaporated to dryness under reduced pressure, and the residue well extracted with hot alcohol (total 15 cc) The extract was filtered, treated with a little Hyflo and filtered through a little Hyflo The filtrate was evaporated to dryness in vacuo under nitrogen at room temperature and the residue left to solidify

The resulting solid, admixed with some oil, was dried on a porous tile, ground up with dry ethyl acetate, filtered off and dried The compound formed a colourless partially crystalline solid, mp 214-215° (decomp), 02 g (yield 68 per cent) (Found C, 346, H, 33 Equiv 845 Loss in weight at 180° with some decomposition, 90 C₁₀H₁₀O₄P₂ 1½H₂O requires C, 346, H, 37 Equiv 868 Loss of 1½H₂O requires 74 per cent)

(b) From crude dibarium salt The dibarium salt (7 879 g) was stirred in water (200 cc) till solution was almost complete and N sulphuric acid added till only a trace of free barium ions remained, the precipitated barium sulphate filtered off through a layer of Hyflo, and the colourless filtrate evaporated to dryness under reduced pressure, leaving 19 g of waxy solid This solid was dissolved in alcohol (150 cc), filtered through Hyflo, and allowed to evaporate at room temperature in vacuo The residue crystallized, mp 210-212° (decomp) (equiv 773) The alcohol treatment was repeated and when most of the alcohol had evaporated, a little ethyl acetate was added The 1 4naphthohydroquinone diphosphate partially crystallized in colourless prisms on standing, m p 214-215° (decomp) (Found, in material dried at 80° C, 369, H, 36 per cent Equiv 796 C10H10O6P2 HO requires C, 36 5, H, 3 4 per cent. Equiv 82 3)

The tetracyclohexylamine salt can also be prepared from the free acid in water by addition of excess cyclohexylamine. The residue after evaporation crystallizes from methyl alcohol, m p 193 5-194°

(decomp) identical with the material prepared from the dibarium salt

1 4-Naphthohydroguinone mono-hydrogen succinate —1 4-Naphthohydroquinone (1 g.) and maleic anhydride (6 g) were carefully mixed and the mixture heated under nitrogen for two hours at 160° molten mass was collected, powdered, and extracted with hot ether (400 cc) in portions of 50 cc ether extract was filtered after 24 hours from undissolved anhydride (3 3 g) and extracted with 1 per cent sodium carbonate (45° cc) in portions of 4×10 cc The first extracts were acid, the last and 1×5 cc extract was amphoteric against litmus The combined 1 per cent sodium carbonate extracts remained clear on acidifying and were discarded extraction with 5 per cent sodium carbonate followed (90 cc in portions of 20, 20, and 5×10 cc) second extract, at once acidified with 5 N sulphuric acid (15 cc), gave an oily, reddish precipitate which The precipitate was dissolved in solidified quickly The red brownish ethereal solution was washed with water, dried over anhydrous sodium sulphate, decolorised with charcoal, and brought to dryness The slightly coloured, crystalline residue (0.7 g) was extracted with hot benzene (20 cc) which removed the coloured impurities completely Yield 06 g. 175-176° (decomp) Recrystallization from ether/n-hexane gave a product melting at 171°, which was again recrystallized from methanol/water It was finally obtained in transparent, irregularly shaped plates, single or arranged in rosettes of tilted plates, mp 167° (decomp) Dissolved in dilute alcohol, the substance gives a purple colour reaction with aq ferric chloride (Found C, 645, H, 435 Equiv 251 C₁₄H₁₂O₃ requires C, 646, H, 465 per cent. Equiv 260)

1 4-Naphthohydroquinone bis-hydrogen succinate -1 4-Naphthohydroquinone (0 5 g) and maleic anhydride (3 g) were brought to reaction as described The molten mass was poured into water The undissolved material was powdered and re-extracted with water (50 cc) for one hour residue was collected, washed and dried in vacuo The dried substance (0.5 g) was dissolved in ether (75 cc) by heating The slightly cloudy solution became clear after being gently treated with norite The norite was removed by filtration and the filtrate concentrated until crystals began to appear crystallization was completed by adding n-hexane in The collected crystals (04 g, mp the usual way 137°) were recrystallized from hot ether (180 cc), discarding small amounts of ether insoluble material The ethereal solution was concentrated to ca 20 cc and allowed to crystallize for 24 hours at room The colourless crystals (0 18 g) so obtained gave no colour reaction with aqueous ferric chloride and seemed to be pure, mp 144.5° were analysed, since preliminary experiments had shown that the substance is decomposed by repeated recrystallization. (Found C, 60.5, H, 46 C₁₈H₁₆O₈ requires C, 600, H, 4.5 per cent 173 Equiv 180)

SUMMARY

The antimitotic properties of maleic acid, citraconic acid, fumaric acid and mesaconic acid have been examined in cultures of chick fibroblasts in vitro Maleic acid proved to be a strong antimitotic

The connection of this group of substances with the 1 4-quinones has been discussed

In the hydroquinone series 1 4-naphthohydroquinone diphosphate has been prepared and its antimitotic properties have been examined. It was found to be 1,000 times as active as 2-methyl-1 4-naphthohydroquinone diphosphate. 1 4-Napthoquinone mono-hydrogen succinate was completely inactive.

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A SIMPLE RECORDING IMPULSE COUNTER

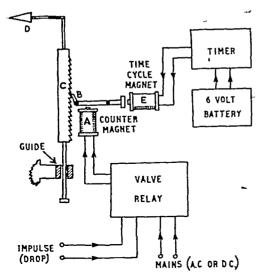
BY

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(Precised March 27, 1948)

During an investigation of the action of drugs upon the isolated mammalian heart by means of the Langendorff preparation, it was observed that certain substances produced a marked acceleration in the rate of the heart beat. In order that such observations could be recorded, an impulse counter was designed capable of making a permanent record on a kymograph



SCHEMATIC DIAGRAM OF RECORDING IMPULSE COUNTER FIG. 1

The principle of the impulse recorder is shown diagrammatically in Fig 1 Each impulse operates the electromagnet A and draws down the pawl B When the circuit is broken at the end of the impulse, the return of the armature pawl lifts the rack bar C by one tooth. There is a second pawl concealed by B in the diagram, which prevents the rack bar from falling while the pawl B is in motion

The rack bar is cut to give 50 teeth each of one millimetre pitch and a writing point D is attached to the top of the bar Impulses are therefore

recorded by a line which rises 1 mm per impulse on the kymograph drum and may be a stepped diagonal line on a fast moving drum, or a virtually vertical line on a slow moving drum. The impulse count is recorded over timed intervals which can be derived from any time marker clock used in physiological laboratories. At the end of the counting cycle an impulse from the time clock energizes the electromagnet E which pulls back the complete armature and pawl assembly so that the rack bar is reset to the base line

The electromagnet used for counting the impulses is wound to a resistance of 1,500 ohms and needs an operating current of 15 milliamps. This has been so constructed for convenience in operating the device from a valve relay of the pattern described by Winton (1936, 1939)

The time cycle release magnet coil is wound to a resistance of 4 ohms and operates on a 6 volt supply, but could be arranged to operate at mains voltage if necessary

The photograph Plate I shows the construction of the device and the same lettering has been used as in the diagram. The two projecting screws F and G enable the counter to be set or released manually

In recording the rate of the isolated heart the impulse was derived from two contacts on the lever recording the individual beats, these contacts were connected to the valve relay unit

The counter can be used for recording a drop outflow with very satisfactory results, by using a silver drop tube of the pattern mentioned by Winton (1936), and connecting this to the valve relay

Several workers have described instruments of this type, but the earliest is probably that of Negrin (1919) followed by the instruments of Fleisch and later that described by Gaddum (1938)

It is common experience in the science of measurement that several instruments are neces-

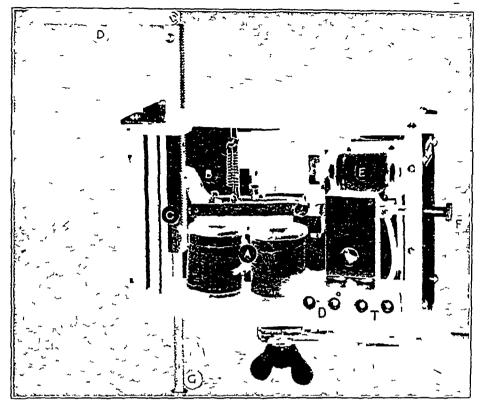


PLATE I -Recording impulse counter with cover removed to show mechanism

sary to cover a wide range of variation in a given function Gaddum's device records on the kymograph the time elapsing between two successive impulses, and is excellent when these impulses are several seconds apart, but unsuitable for impulse rates faster than 100 per minute. The instrument at present described counts the impulses and by alterations in the slope of the recorded trace gives an indication of a change of interval between impulses within any one timing cycle, which can be of any length greater than 5 seconds. The fastest rate the present model can record is between 550 and 600 impulses per minute.

The applications of such a device are many, and its fundamental advantage is its inherent linearity and the ease with which the individual impulses can be counted on the record

I should like to thank Mr Ellis, of Messrs C. F Palmer (London), Ltd, who constructed the instrument illustrated from a simple prototype

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SYSTEMIC EFFECTS OF ADENOSINE TRIPHOSPHATE

BY

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The experiments described in the present paper were the outcome of an incidental observation made in decerebrate cats when adenosine triphosphate (ATP) was injected into the artery supplying a leg muscle, the tibialis anticus These injections were intended to produce the direct muscle contracting action of ATP first described by Buchthal and Kahlson (1944) We started with small doses of ATP which gave no contractile response, the dose was then increased with the result that sometimes, but by no means always, immediate weak contractions could be recorded from the tibialis anticus, but the injections produced additional and more striking effects After a latency of some 30 seconds, the time necessary for the ATP to reach the general circulation after having passed through the tibialis anticus muscle, respiratory changes occurred, followed by strong contractions of the skeletal muscles of the limbs and the whole body of the animal ATP is known to produce profound changes in the cardio-vascular system (Drury and Szent-Gyorgyi, 1929, Gaddum and Holtz, 1933, Gillespie 1934, McDowall, 1944, Bielschowsky, Green and Stoner, 1946) and the possibility was therefore envisaged that these general effects might be the outcome of circulatory events

The present paper deals with circulatory, respiratory, and other systemic effects of ATP when injected into different parts of the circulatory system

METHODS

Most experiments were performed on cats, decerebrate or in chloralose anaesthesia, a few experiments were performed on dogs and rabbits also anaesthetized with chloralose. The systemic arterial blood pressure was recorded from the left external carotid or the femoral artery with a mercury manometer. In the same way the pressure in the pulmonary artery was recorded in some cats as well, the artery of the lower lobe on the left lung being

cannulated in this case The heart rate was read from the tracing of the systemic blood pressure, for this purpose the drum was run at a fast speed

For intravenous injections of ATP cannulae were tied into a jugular or femoral vein When the ATP was intended to reach the systemic, without first passing the pulmonary circulation, it was injected either in o the left auricle or through a glass cannula tied into the left auricle or through a long fine syringe needle, with a blunt tip, which was passed through an opening in the right external carotid or subclavian artery down to the base of the ascending Heparin was often given in these experiments For injecting ATP into the brain circulation it was injected into the vertebral or external carotid artery For the injections into the vertebral artery again a fine blunt needle attached to a syringe, filled with the amount of ATP to be injected, was passed through an arterial side branch into the vertebral artery. In order to ensure that all the injected ATP passed down the vertebral artery the vessel was sometimes tied over the needle during the injection, but immediately afterwards the ligature was loosened and the needle withdrawn so that the blood supply to the vertebral artery was restored When ATP was injected into the external carotid, the carotid sinus region was usually denervated and the injections were made through a cannula tied into the lingual artery pointing toward the carotid which during the injections was kept clamped at its aortic end with an arterial clip

When we began injecting ATP into the left coronary artery we used cats and introduced a fine olunt syringe needle slightly bent at its end, through an opening in the subclavian artery into the ascending aorta and then guiding it by touch into the mouth of the left coronary artery. It was either kept here without further fixation during the injection or fied with a thread previously placed loosely round the origin of the coronary artery. In these experiments the left coronary artery is supplied with blood by collaterals only Later on we used in cats and dogs the methods described by Dawes for cats (1947) With this method the circulation through the left coronary artery is not interrupted A specially shaped glass cannula is required for these experiments, it was kindly supplied to us by Dr G S

^{*} With a grant from the Swedish Medical Research Council

[†] With a grant from the Medical Research Council

Dawes (Oxford) In all experiments with injections anto the left coronary artery heparin was used

Respiration was recorded by a modification of Gaddun's (1941) method. The trachea was attached to a glass tube with inflow and outflow valves, which gave as little resistance to respiration as possible. The expired air was blown into a 10 litre bottle, from which a fine glass tube led to the outside air and a wide tube to a sensitive tambour for recording the respiratory changes. Unlike Gaddum's method this arrangement does not provide a quantitative record of the respiratory changes, but it serves the purpose of recording easily changes in rate and depth of respiration. Inspiration is shown in the tracings as a downward, expiration as an upward stroke.

In some experiments it was desired to exclude vagal impulses for short periods. For this purpose the vagi were cooled at the neck by placing them each on a separate 15 cm wide thin copper plate, which was slightly bent at its end in order to prevent the nerves from slipping off the plate. The plates were 16 cm long and kept cold during the period of cooling the nerves by placing the free ends into beakers full of crushed ice. In addition a little metal box was soldered on each plate near the end provided to take the nerve and also filled with crushed ice.

Three different preparations of the barium salt of ATP were used One sample was prepared by ourselves by a mocified version of Lohmann's method (1931) one sample was kindly supplied to us by Professor F Buchthal (Copenhagen), and a third sample was obtained from Boots' Drug House Before use the Ba salt was converted into the When the three preparations were comsodium salt pared for their ability to activate the synthesis of acetylcho ine in extracts of acetone dried brain tissu the sample obtained from Boots was found to be only 80 per cent as active as the other two samples, which had practically identical activity obvious difference was observed with the three preparations on injections into the circulations of cats or dogs, a 20 per cent reduction in activity, however, would not have been noticed in these experiments In the text the dose of ATP injected has been expressed as ATP pyro—P (ATP—P) In order to ob ain the value for the total phosphorus injected the value has to be multiplied by 15 In a few experimen's control injections were made with creatine phosphate which was kindly prepared for us by Dr P Eggleton and Dr Nimmo Smith as the synthetic Ba salt and converted before use into the Na salt The values for creatine phosphate are expressed in the text as mg total labile phosphorus (CrP-P), about 1 mg of which is present in 12 mg of the Ba salt

RESULTS

Circulatory effects in cats

The intravenous injection of 02 to 04 mg of ATP—P causes a profound and steep fall in arterial blood pressure (Fig 1) During the fall

the heart pulsations on the blood pressure tracing become feeble or the float on the mercury mano meter may even write a smooth line on the smoked drum. When the oscillations are not obliterated, pronounced slowing of the heart can be seen on the blood pressure tracings. In Fig. 1a the steep fall in arterial blood pressure is associated with an initial period in which the heart oscillations are no longer visible on the tracing, later pronounced slowing of the heart will be seen. In the experiment of Fig. 1b the blood pressure tracing gives during the whole period of the depression a smooth line.

When the blood pressure has recovered a renewed injection of ATP produces approximately the same strong fall in arterial blood pressure as the first injection, but on further repetition of the injections the sensitivity to 'ATP gradually de creases, so that it has to be given in increasing doses in order to elicit responses as strong as the initial ones

Other organic as well as inorganic phosphate compounds injected in doses containing the same or even a greater amount of total phosphate than that injected with the ATP, produced either no fall in arterial blood pressure (for instance 0 6 mg CrP—P, 7 mg sodium triphosphate or 15 mg sodium pyrophosphate) or a fall uncomplicated by slowing of the heart or by reduction in the heart oscillations (for instance 10 mg muscle or yeast adenylic acid)

The following factors are responsible for the depressor action of ATP in cats (a) obstruction in the pulmonary circulation, causing a reduction in cardiac output, (b) reflex slowing of the heart causing further reduction in cardiac output, and (c) vasodilatation

Obstruction in the pulmonary circulation—In order to observe the effect of ATP on the pulmon ary circulation it is best either to cut the vagi or to give atropine in order to eliminate the slowing of the heart. Under these circumstances ATP still produces its pronounced depressor effect, showing that the slowing of the heart observed in the absence of atropine and with the vagi intact is not the sole or main factor responsible for the fall in systemic blood pressure produced by ATP

The injection of 02 to 04 mg ATP—P into the jugular vein or into the right auricle of a cat with the thorax open, artificial ventilation and the vagi cut or atropine given, produces tremendous swelling of the pulmonary artery and of the whole right heart, ventricle and auricle, the left remains small. This can be observed with the naked eye in a cat with open thorax and artificial ventilation. These volume changes do not take place when the

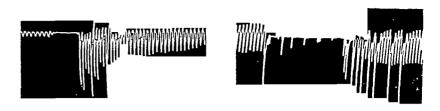


Fig 1—Respiration (upper) and arterial blood pressure (lower) tracings of decerebrate cat (a) and cat under chloralose (b) At A, 02 mg, at B, 04 mg ATP-P intravenously Time in 10 seconds

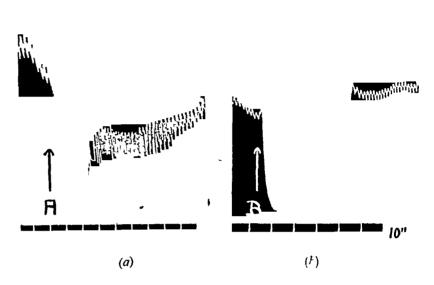
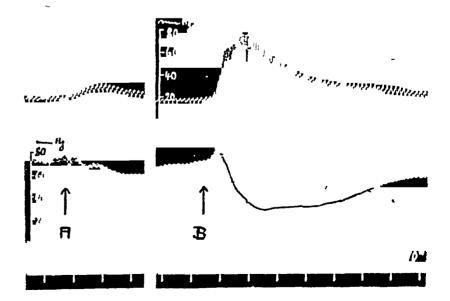


Fig 2—Pressure in pulmonary artery (upper tracing) and carotid (lower tracing) of 2 2 kg cat under chloralose Thorax opened, artificial ventilation, both vagi cut At the arrows 0 2 mg ATP-P into the left auricle (at A) and into the jugular vein (at B) Time in 10 seconds



same dose of ATP is injected into the left auricle and reaches the pulmonary artery greatly diluted after its passage through the systemic circulation. Incisions into the carotid arteries, when the intravenous injection of ATP had produced its characteristic fall in arterial blood pressure with elimination of the heart oscillations and the typical many changes in heart volume, caused very little bleeding, whereas a subsequent incision into the right auricle or the superior vena cava produced a rushing out of blood into the thorax. All these observations are accounted for by a strong obstruction in the pulmonary circulation whereby the blood is prevented from reaching the left heart, consequently the cardiac output drops.

In Fig 2 the pulmonary obstruction produced by ATP is shown by recording its effect on the pressure in the pulmonary artery simultaneously with the carotid blood pressure Usually the opening of the thorax and tying a cannula into a pulmonary artery and into the left auricle lowers the carotid blood pressure to about 100 mm Hg or to an even lower level In this condition ATP apparently produces little further vasodilatation, as seen from the fact that an injection of 02 mg ATP—P into the left auricle causes scarcely any further fall in carotid blood pressure (Fig 2,A) The pressure in the pulmonary artery_also is little affected by this injection of ATP The result, however, is different when the same dose of ATP is injected intravenously (at B), the pressure in the pulmonary artery rises immediately and steeply from its initial level of about 20 mm. Hg to about 80 mm Hg The pressure in the carotid artery starts to fall a second or two after the beginning of the rise in the pulmonary artery and then follows it in reverse direction During the fall in systemic blood pressure the heart oscillations on the carotid pressure tracing become feeble

With repeated intravenous injections of the same dose of ATP the effect on the pulmonary blood pressure becomes gradually smaller and so does the fall in systemic blood pressure. But an increase in the dose of ATP produces again the strong pressure changes in the pulmonary and carotid artery

Vasodilatation in the systemic circulation—We have seen that in cats in which a cannula has been tied into the pulmonary artery the arterial blood pressure often falls to a level of about 100 mm Hg or lower owing most likely to a decrease of tone in the systemic vessels. In such cats no evidence could be obtained for a vasodilator action of ATP. The result, however, is different in those cats in which the level of the arterial blood pressure has remained high even after opening the

thorax and tying a cannula into the left auricle, in these conditions ATP in doses too small to produce a fall in arterial blood pressure, when injected in travenously, has such an action when injected into the left auricle. In the experiment of Fig 3 a dose of 0.05 mg ATP-P injected intravenously

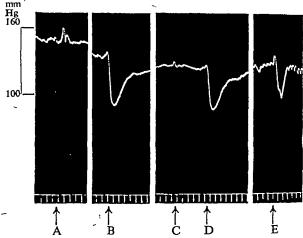


FIG 3—Carotid blood pressure of 3 2 kg cat under chloralose Both vagi cut, thorax opened, artificial ventilation At A and E intravenous injection of 0.05 mg and 0.2 mg ATP-P respectively At B and D 0.025 mg ATP-P and at C 1 c.c. saline injected into the left auricle Time in 10 seconds.

at A did not lower the arterial blood pressure, but half the amount injected into the left auricle, at B and D, caused an evanescent but pronounced fall, which in fact was stronger than that produced by eight times the dose of ATP injected intravenously at E. Vasodilatation in the systemic circulation therefore also contributes to the usual depressor action of intravenous injections of ATP.

No experiments have been carried out to analyse the vasodilatation. It is unlikely that reflex vaso dilatation occurs with ATP when the vagi nerves have been cut. This may happen, however, in cats with intact vagi which could carry afferent impulses to the vasomotor centre. The effect would then resemble that of veratrin (Dawes, 1947). Our experiments also do not deal with the possibility of a centrally induced vasodilatation. Such an effect cannot be excluded by our finding that ATP, at least in cats under chloralose anaesthesia, has no central cardiac action.

Slowing of the heart—The pronounced slowing of the heart when ATP is injected intravenously is dependent on the integrity of the vagi. This had been observed by McDowall and by Bielschowsky, Green, and Stoner (1946) They had concluded that ATP stimulates the vagus centre in the medulla

Our results, however, show that the slowing is mainly accounted for by a reflex, the afferent and efferent impulses of which are carried in the vagus In fact in cats under chloralose anaesthesia the bradycardia obtained on injections of 0 2-0 4 mg ATP-P-is solely accounted for in this In decerebrate cats, on the other hand, stimulation of the cardio-inhibitor centre by ATP probably contributes to the bradycardia In these cats injections of 02 to 04 mg ATP-P into a vertebral artery slow the heart, but in cats under chloralose neither an injection into a carotid artery, in central direction, nor into a vertebral artery was effective, whereas the same amount of ATP injected into the left auricle produced pronounced bradycardia even when the carotid and vertebral arteries were occluded during the injec-It might be mentioned in this connexion that clamping of the vertebral arteries in decerebrate cats, but not in cats under chloralose, also led to bradycardia The reflex nature of ATPbradycardia in cats under chloralose anaesthesia is illustrated by the experiment (Fig 4) in which 0.1 mg ATP-P was injected either into the right vertebral artery (at B) or into the left auricle (at A) The injection into the auricle reduced the heart rate from 34 per 10 seconds to 9 per 10 seconds, whereas the vertebral injection did not change it Between B and C the carotid and vertebral arteries were clamped, this caused a rise in blood pressure but no slowing of the heart. When the blood pressure had reached a relatively steady level 0.1 mg ATP-P was again injected into the left auricle, The bradycardia was not prevented by having excluded the brain circulation, but was abolished after cutting the vagi In another experiment on a cat in chloralose anaesthesia 01 mg ATP-P was injected into the right carotid, whilst it was kept clamped for a few seconds at its aortic end, or into the left auricle, the carotid and vertebral arteries being kept occluded for about 10 seconds before and after the injection Again only the injection into the left auricle caused slowing of the heart (from 34 to 15 beats per second) despite the fact that the pathway to the brain was occluded, but the bradycardia was abolished after cutting the vagi This experiment shows in addition that the reflex does not originate from chemoreceptors in the carotid body, a fact which has been verified in cats with both carotid sinus nerves ligated and cut. These experiments do not exclude an action of ATP on the chemoreceptors in the aortic body, but it would be strange if they were, and those in the carotid body were not, sensitive to ATP The region of the aortic arch, however has not been excluded as a contributory area from which the reflex could be elicited although, to some extent at least, it originates in the heart itself and thus resembles the bradycardia produced by veratrin alkaloids (Dawes, 1947), this was evident when ATP was injected into the coronary artery

In one cat we succeeded in tying a needle cannula, introduced through the subclavian artery, into the main branch of the left coronary artery without causing ventricular fibrillation Fig 5 is taken from this experiment, the injection of 05 cc of saline into the coronary artery had no effect (at A), but 0.1 mg ATP-P slowed the heart rate from 28 per 10 seconds to 13 per 10 seconds The effect of ATP could be obtained with each renewed injection, but only as long as the vagi were left intact A post mortem injection of Chinese ink through the cannula into the coronary showed that it supplied practically the whole left heart There must, however, have been effective anastomoses with other coronary branches which kept the muscle supplied with blood Some indication of these was obtained during the experiment by the fact that there was a strong back flow of blood whenever the syringe attached to the needle was removed after an injection. In later experiments Dawes's method was used in which the coronaries are continuously supplied with blood In these experiments also the injections of ATP into the coronary circulation caused slowing of the heart, but not after cutting the vagi Doses larger than 0.2 mg ATP-P were The slowing produced with the vagi intact was often less pronounced than that following the injection of a similar dose of ATP low This difference down into the ascending aorta may be due to the fact that only part of the heart area from which the reflex is elicited will be reached when ATP is injected through the cannula tied into one coronary or that some receptors for this reflex are situated in the aortic arch

Slowing of the heart by ATP in rabbits and dogs—Our results obtained in rabbits are in agreement with those of Bielschowsky et al (1946) who found that in these animals ATP has a direct cardiac depressant action. The injection of ATP into the left auricle caused pronounced bradycardia which was not prevented by cutting the vagi or by atropine. The effect was evident with 0.05 mg ATP—P and became stronger with larger doses, but on repeated injections the slowing became gradually less pronounced.

In dogs the injections of ATP into the left auricle or down to the base of the ascending aorta also caused pronounced slowing of the heart, which, like that seen in cats, was prevented by

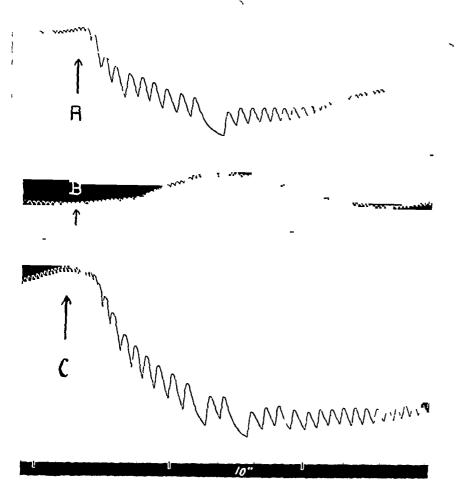


Fig 4—Carotid blood pressure of 32 kg cat under chloralose, thorax open Artificial respiration. At A and C 01 mg ATP-P injected into left aunce, at C with carotid and ver tebral arteries occluded At B 01 mg ATP-P injected into left vertebral artery Time in 10 seconds

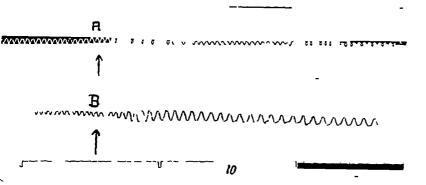


Fig 5—Blood pressure from femoral artery of 2 kg cat under chloralose. Thorax open Artificial ventilation Heparin Cannulatied into left coronary artery through subclavian artery At A injection of 0.5 c c saline, at B of 0.1 mg ATP-P into coronary artery Art blood pressure about 60 mm Hg Time in 10 seconds.

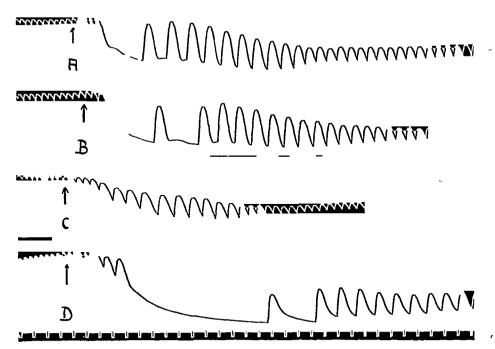


FIG 6—Carotid blood pressure of 11 kg dog under chloralose Thorax open, artificial ventilation At A and B injections of 0 2 mg ATP-P into ascending aorta at its base and at C and D into right vertebral artery At B carotid and vertebral arteries occluded Time in seconds

cutting the vagi or by atropine The effect was partly reflex, partly central in origin In the experiment of Fig 6, for instance, 02 mg ATP-P

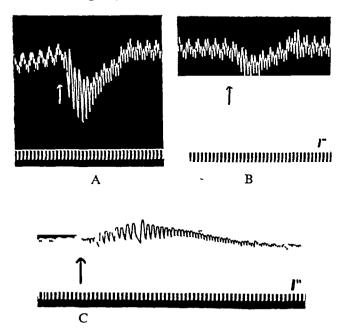


FIG 7—Arterial blood pressure of 8 kg dog under chloralose Injections of 0.2 mg ATP-P into right carotid artery through cannula in lingual artery (at A and B) and into left coronary (at C) Between A and B mass ligation of right occipital, internal carotid and other small arteries leading to brain, but leaving sinus nerve intact Between B and C thorax opened, artificial ventilation, hepatin, cannula tied into left coronary artery for perfusion Time in seconds

injected low down into the ascending aorta produced pronounced bradycardia whether the carotid and vertebral arteries were left open (at A) or clamped (at B) before and during the injection. The effect therefore cannot be wholly due to sumulation of the cardio-inhibitor centre by ATP The participation of a central factor, however, was shown by the bradycardia obtained when the same dose of ATP was injected into a vertebral artery, the slowing of the heart was weaker at C but stronger at D than that produced by injections into the left auricle at A and B These doses of ATP had certainly no direct cardiac depressant action since they were ineffective after cutting the vagi In another experiment central cardiac inhibition was obtained by injecting 0.2 mg ATP-P into the carotid through a cannula in the lingual artery whilst the carotid was clamped at its aortic end Ligating and cutting the sinus nerve did not abolish or diminish the effect, but if, subsequently the vessels from the carotid leading to the brain were tied ATP became ineffective The reverse procedure was adopted in the experiment shown in Fig 7, the bradycardia became nearly abolished after the blood vessels to the brain had been tied, although the sinus nerve was kept intact. This was done between A and B The slight slowing seen at B is probably not due to ATP, since saline injections into the carotid artery with its lower end occluded produced the same effect. The injections probably increased the pressure in the carotid artery for a short period and consequently stimulated the pressor receptors

Inhibition of the sympathetic is not involved in the reflex slowing of the heart, since the removal of the stellate ganglia did not diminish the effect

The mechanism of the reflex bradycardia appears to be similar to that in cats and originates, at least to some extent, from the heart itself. In a few experiments the left coronary artery was perfused and an injection of ATP into it produced bradycardia provided the vagi were not cut (Fig 7 C)

Respiratory changes in cats

An intravenous injection of 01 to 04 mg ATP-P produces profound changes in pulmonary ventilation. There is often immediate cessation of movements lasting for 10 to 50 seconds. The apnoea precedes the fall of arterial blood pressure or occurs simultaneously. In some experiments apnoea was absent, instead there was a period of shallow frequent respiratory movements. Both changes might be the sole effects or they might be followed by strong hyperventilation with increased-

Fig 8—Record of respiration from four cats A and D under chloralose, B and C decerebrate Effect of intravenous injections of 0.1 mg (in A and C) and 0.2 mg (in B and D) of ATP-P Time in 10 seconds shown on top of the figure for A, B and C and at the bottom for D

depth and frequency of breathing and sometimes incomplete expiration. Some of these changes are illustrated in Fig. 8 at A there is a period of apnoea, at D a period of shallow frequent breathing in cats under chloralose, at B and C there are periods of apnoea followed by strong hyperventilation, which at C occurs with incomplete expiration, these tracings are from decerebrate cats Injections of creatine phosphate (0 6 mg CrP-P), adenylic acid (10 mg) or sodium pyrophosphate (10 mg) do not produce similar changes in pulmonary ventilation

A complete analysis of the respiratory changes has not been carried out. The results so far obtained indicate, however, that several mechanisms are involved. ATP has a direct action on the respiratory centre and, in addition, seems to affect the centre indirectly by reflexes originating in the lungs, the impulses being carried via the vagi. No experiments have been performed to find out if it affects the centre also via the chemoreceptors.

The initial period of apnoea is brought about, partly at least, by a reflex via the vagus, partly it

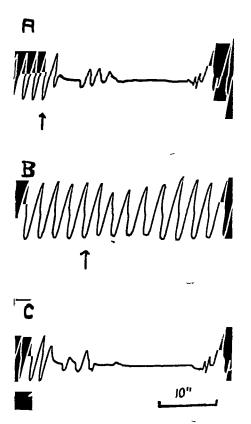


FIG 9—Record of respiration from 3 kg cat under chloralose At A, B and C intravenous injection of 0 4 mg ATP-P At B both vagi cooled at the neck Time in 10 seconds

may result from a central action of ATP Cutting the vagi certainly reduces and sometimes even abolishes the apnoea A certain caution, however, is necessary in the interpretation of this result, since repeated injections of ATP may cause reduced effects on respiration even without cutting the vagi. This argument does not apply to those experiments in which the vagi were cooled and the effect was found to be reversible. A striking experiment of this kind is illustrated in Fig. 9

Some evidence in favour of the conception that the apnoea, as far as it is brought about reflexly, is due to stimulation of afferent fibres in the lungs, is given by an experiment in which the effect on respiration of 0.2 mg ATP-P injected intravenously and into the ascending aorta has been compared. Instead of the initial apnoea produced by the intravenous injection there is a period of irregular breathing on arterial injection and, after cutting the vagi, the intravenous injection also produced these changes only

A direct effect of ATP on the respiration centre is assumed from the fact that cutting the vagi usually did not wholly eliminate the respiratory changes and from the effects obtained when ATP was injected into a vertebral or carotid artery with denervated carotid sinus region. These injections produced respiratory changes, but they were weaker than those obtained with the same dose of ATP injected intravenously and with intact vagi

Muscular contractions in cats

As mentioned earlier the experiments on circulation and respiration with ATP were the outcome of an incidental observation i.e., widespread muscular contractions. These were obtained regularly in decerebrate cats on intravenous injection of 0.2 to 0.4 mg. ATP-P, but in cats under chloralose anaesthesia larger amounts of ATP had to be injected in order to produce this effect and even then it was not obtained regularly or in as pronounced a form as in decerebrate cats, only the latter therefore were used for further analysis.

The contractions consisted of a typical pattern After a latency of between 15 to 20 seconds the forelegs became maximally extended and extreme opisthotonos developed. Sometimes the hind legs too became strongly extended. In several experiments, particularly when the cat was lying on its side with the head elevated, co-ordinated running movements occurred especially in the forelegs. Micturition, defaecation and strong peristals sometimes occurred. A similar pattern of muscular contractions to that observed after ATP could be elicited in the decerebrate cat on occluding the vertebral arteries with arterial clips, this was

followed within less than 10 seconds by rigid extension of the forelegs and strong opisthotonos It appeared possible therefore that anaemia of the central nervous system, caused by the fall in arterial blood pressure or stoppage of respiration, was responsible for the ATP contractions But the effect occurred also when the depressor action of ATP was greatly reduced by injecting the ATP into the left ventricle, after atropine and with artificial ventilation Central anaemia may, however be a contributory factor when ATP is injected in-When injected into the left ventricle the muscular contractions start after a shorter latency than after intravenous injections observation excludes also a reflex action of ATP originating in the lungs as the cause for the muscular effects They occur also when the vagi nerves have been cut In two out of five cats, however, the dose of ATP necessary to produce the muscular contractions on intravenous injection had to be doubled after the vagi were cut, thus afferent impulses in the vagi may perhaps participate to some extent in the initiation of the muscular contractions Essentially, however, they are the result of an action of ATP on the central nervous system and therefore easily obtained when ATP is injected into a vertebral artery, when this is done the latency is a few seconds only In decerebrate cats an action of ATP on supraspinal levels is probably the main cause of the muscular contractions, but ATP has a stimulating action also on the When the spinal cord was trancervical cord sected just above or below the origin of the first cervical nerve roots the injection of 02 to 03 mg ATP – P into a vertebral artery produced muscular contractions but of a pattern different from that seen in the decerebrate cat The main effect was on the hind legs and resembled the pattern of a typical scratch reflex on one or both legs Rapidly alternating flexion and extension occurred at the ankle, the knee and less at the hip. In fact some flexion at the hip was more or less maintained during these rhythmic movements. The effect usually started on the side where the ATP was injected and if weak the movements occurred only at the ankle or at the ankle and knee When the effect was pronounced the feet of both hind legs were brought to the shoulder region and exerted here the typical scratching movements The effect on the forelegs was less pronounced and consisted of fine clonic movements and extension. A similar pattern of muscular contractions was produced in these cats on clamping one or both vertebral arteries contractions produced in these cats, even those on the hind limbs, resulted from an action of ATP on the cervical cord, because no effect or slight muscular contractions only were observed when the same dose of ATP was injected into the descending aorta or into the central end of the superior mesenteric artery with the iliac arteries clamped during the injections With larger doses (over 0.4 mg ATP—P), these injections caused muscular contractions but after a latency of more than 30 seconds, i.e., after the ATP had passed the lower body circulation, the legs and the left heart. The strong congestion of the pulmonary artery and the right heart preceding these contractions was good evidence that the ATP had reached the pulmonary circulation in effective concentration.

DISCUSSION

The symptomatology of ATP when injected intravenously into cats is a complex one including peripheral, reflex and central mechanisms

The profound and steep fall in arterial blood pressure is accounted for to a great extent by obstruction in the pulmonary circulation leading to a diminution in cardiac output Gaddum and Holtz (1933) have described the constrictor effect of ATP on the pulmonary arteries, but its decisive role for the depressor action of this substance has not been recognized. According to these authors other phosphate compounds show the effect to a smaller degree, and in other animals, at least in the - dog, the pulmonary vessels are less sensitive to ATP than in the cat It would be of interest to know if in the dog the smooth muscles of the hepatic veins show instead a special sensitivity to ATP A second direct effect contributing to the depressor action of ATP in cats is the vasodilatation it produces in the systemic circulation is also the possibility, mentioned before but not yet examined, of vasodilatation brought about by a reflex and central action of ATP and inhibiting the sympathetic tone A third factor which will accentuate the depressor action of ATP is a bradycardia sufficiently strong to cause reduced cardiac output The bradycardia is to some extent only accounted for by a central action of ATP, the explanation given by McDowall and by Bielchowsky et al. because it is abolished by cutting the vagi procedure, however, does not exclude the possibility of a reflex action of ATP We could in fact show that ATP stimulates afferent fibres of the vagus in the heart and thus reflexly produces bradycardia The effect resembles that produced by veratrin alkaloids (Dawes, 1947) In cats under chloralose this reflex mechanism accounts for the whole effect, but in decerebrate cats in which the centre appears to be particularly sensitive to ATP and in dogs under chloralose anaesthesia central actions of ATP con-

tribute to the bradycardia According to Mc-Dowall and to Bielschowsky et al ATP has in cats also a direct depressant/effect on cardiac muscle. which is not abolished after section of the vagi These authors, however, have not excluded the possibility that changes resembling those produced by a direct cardiac depression may easily be simulated by obstruction in the pulmonary circulation with its consequent engorgement of the right and insufficient blood supply to the left heart. In those experiments in which we injected ATP into the left heart and thereby avoided pulmonary obstruction no signs of depression of the heart muscle were seen The position is different in rabbits in which depression of the cardiac muscle occurs with ATP and has been demonstrated in isolated perfused hearts

The profound changes in respiration produced by ATP have, so far as we know, not been described previously. A more detailed analysis than we have performed would be necessary in order to evaluate the different mechanisms involved in this effect. As far, however, as the results go, they show that ATP affects the respiratory centredirectly as well as reflexly, via impulses in the vaging probably originating in the lungs.

The muscular contractions observed on injections of ATP intravenously or into the left heart are central effects of ATP They may be accentuated by the circulatory and respiratory changes causing central anaemia, because a similar pattern of muscular movements could be obtained on They may be occluding the vertebral arteries further accentuated by afferent impulses in the vagi, because in two cats at least the dose of ATP had to be increased after vagotomy in order to This might be elicit the muscular contractions explained, however, simply by the fact that the vagal slowing of the heart is one of the circulatory events which leads to the central anaemia

Buchthal, Engback, Sten-Knudsen, and Thomasen (1947) were the first to describe centrally produced muscle contractions when ATP was injected into a vertebral artery of a chloralosed cat They recorded action potentials from the muscles of the forelegs and attributed the effect to a stimulation of the anterior horn cells in the cervical cord, since in their experiments the vertebral arteries were occluded at the level of the atlas and injections of Indian ink had shown that only the cervical segments were stained We feel unable in our experiments to localize or to confine the stimulating action of ATP to these cells The pattern of muscular movements varied in decerebrate and in spinal cats, but was the same whether produced by ATP or by occlusion of the vertebral artery In

decerebrate cats a postural pattern was obtained similar to that seen when the head in these animals is strongly dorsiflexed i.e., extensor spasm of the forelegs and opisthotonos Such a pattern of muscular contractions suggests the possibility of a stimulating action of ATP on supraspinal levels In spinal cats with the section just below the medulla oblongata or even at its lower end the pattern of muscular movements produced by ATP as well as by occlusion of the vertebral arteries resembled the scratch reflex If the blood supply of the vertebral artery in these animals were, limited to the cervical cord, this would indicate a representation of this reflex pattern in the cervical It is interesting to note in this connection cord that injections of ATP into the descending aorta produced no or scarcely any contractions of the skeletal muscles, suggesting a special susceptibility of the cervical region of the cord to ATP

The fall in arterial blood pressure, the reflex slowing of the heart, the obstruction in the pulmonary circulation, the vasodilatation, the peristalsis, micturition, vomiting, defaecation, and the muscular contractions of spinal and supraspinal origin, all this complex symptomatology is one which cannot be reproduced by other phosphate compounds, although they may have one or another action in common with ATP other hand this symptomatology is not confined to For instance a striking similarity is found, at least in cats, with the effects produced by serum or plasma The toxic actions of serum in cats were first examined by Brodie (1900) His records of the changes occurring in blood pressure and respiration are undistinguishable from the tracings we obtained with ATP In cats, serum or plasma also produce a pronounced bradycardia, which was shown by Brodie to be of reflex origin, the reflex according to his analysis being initiated in the lungs A re-examination of the effect, however, has shown that the heart is to some extent at least the site from which the reflex originates (Dawes and Feldberg, 1948) Like ATP, serum produces in cats strong contraction of the pulmonary vessels and on its intravenous injection this action may lead to the disappearance of the heart oscillations on the arterial blood pressure tracing and is in fact considered to be the main cause of the fall in pressure (Reid Bick, 1942) Gilding and Nutt (1944) have further observed on injection of stored plasma into cats peristalsis micturition vomiting defaecation, and muscular contractions of a pattern similar to that seen with ATP They obtained the contractions only in decerebrate and not in chloralosed cats but we have seen that ATP also was much more effective in this respect in a decerebrate cat. According to Gilding and Nutt the contractions are abolished by cutting the vagi and would therefore be of reflex origin. The contractions produced by ATP are of central origin however in some experiments section of the vagi necessitated a doubling of the dose of ATP in order to elicit the effect, and if the dose had not been increased the impression might easily have been gained of a reflex nature of the muscular contractions. The experiments with serum thus need re-examination in the light of these findings

One might be tempted from such a close similarity of action to assume that serum and stored plasma owe their action to the presence of ATP, this, however, is not so, otherwise the "toxicity' of serum and plasma would not be confined to cats because ATP exerts its actions also in other Adenylic compounds have been considered as the cause of the toxic action of serum or plasma According to Zipf (1930), adenylic acid is identical with the "Fruhgift" of serum, but adenylic acid cannot produce the toxic effects in All the evidence available suggests in fact that the principle which makes serum and plasma so toxic for cats is a non-dialysable substance and probably a protein of the albumin class (Brodie, 1900, Reid and Bick, 1942, Gilding and Nutt, 1944) There is no evidence to suggest that ATP is linked to a protein constituent in serum and that in this linkage it would be active in cats but not in other animals

SUMMARY

- 1 In cats under chloralose or in decerebrate cats the intravenous injection of 0.2 to 0.4 mg ATP-P causes (a) a steep fall in arterial blood pressure due to constriction of the pulmonary vessels, bradycardia, and vasodilatation, and (b) profound changes in respiration. In decerebrate cats these injections, in addition, regularly produce muscular contractions and often peristalsis, defaecation, vomiting, and micturition. In cats under chloralose anaesthesia ATP produces these additional effects only when given in larger doses and even then not regularly. The complex symptomatology of ATP cannot, or can in parts only, be reproduced by other phosphates.
- 2 The effect of ATP on the pulmonary vessels may be so strong that only little blood can enter the left heart. There is consequently a great reduction in cardiac output, which is to a great extent responsible for the steep fall in arterial blood pressure and which may cause obliteration of the heart oscillations on the blood pressure tracings.

- 3 The strong bradycardia produced by ATP, which causes a further reduction in cardiac-output, results mainly from stimulation of afferent vagus fibres in the heart. This reflex mechanism accounts wholly for the bradycardia obtained in cats under chloralose. In decerebrate cats as well as in dogs under chloralose a central action of ATP contributes to the bradycardia seen after intravenous injections of ATP. On the other hand in rabbits the bradycardia produced by ATP is a peripheral effect.
 - 4 The changes in respiration produced by ATP in cats consist of an initial period of cessation of respiration or of shallow frequent respiration often followed by a period of hyperventilation. ATP affects the respiratory centre directly and indirectly through impulses carried via the vagi and probably originating in the lungs.
 - 5 The muscular contractions obtained in decerebrate cats on intravenous injections of ATP are of central origin and therefore also obtained on injection into a vertebral artery. They may be accentuated, however, by afferent impulses in the vagi and by central anaemia as a result of the cir-

culatory and respiratory effects. The muscular contractions consist in the main of extensor spasm of the forelegs and episthotonos. In spinal cats with the cervical cord intact, intravenous or intravertebral injections of ATP cause muscular contractions, particularly in the hind limbs, of a pattern resembling the scratch reflex.

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THE MAINTENANCE OF A FILARIAL INFECTION (LITO-MOSOIDES CARINII) FOR CHEMOTHERAPEUTIC INVESTIGATIONS

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The purpose of the paper is to describe the maintenance in the laboratory of a filarial infection (Litomosoides carini) which is suitable for chemotherapeutic and other types of investigations. The strain is kept in cotton rats (Sigmodon hispidus) and with one interruption it has now been maintained in our department for 2 years, during which time over 600 rats have been infected as shown by the presence of microfilariae in their peripheral blood

HISTORY

Cotton rats began to be used in the United States for laboratory experiments about 1940, they were employed particularly in the study of viruses and rickettsiae During more recent years these animals have been bred on a large scale in laboratories both in America and in Britain Most of the rats used in the earlier work were wild ones which had been trapped in certain parts of Florida and Texas Many of these wild rats were found to contain a filarial worm, Litomosoides carinii, which had previously been described in these and various other hosts by Travassos (1919), Mazza (1928), Chandler (1931), Ochoterena and Caballero (1932), Vogel and Gabaldon (1932), Chitwood (1933), and Vaz (1934), these are reviewed by Culbertson and Rose (1944) showed that these spontaneous infections in wild rats were very convenient for experiments on the chemotherapy of filariasis, and much work was carried out by these authors (summarized by Culbertson, 1947) and by many other investigators in America holding OSRD contracts The interest thus excited led to a determined search for the arthro-Eventually, Williams and Brown pod vector (1945) showed that the worm developed in the tropical rat mite Liponyssus bacoti Hirst and transmission to clean rats was demonstrated by these workers (1946) and by Scott and Cross (1946) Descriptions of the morphology and life cycle of this mite have been given by Hirst (1913, 1914), Holdaway (1926), Dove and Shelmire (1931, 1932), and others At the end of 1945 Dr R W Williams, Dr J A Scott, and other investigators kindly told one of the authors (FH) their latest results and demonstrated their methods for handling cotton rats-Dr Williams and Dr Scott kindly provided infected rats and mites which were brought back to Britain in February, 1946 Without their generous co-operation this work could not have been started Further supplies of mites were kindly provided at a later stage by Dr Cuckler, of Minneapolis, and by Prof R M. Gordon, of Liverpool The objective of the work here described has been the development and practice of transmission on a large scale so as to obtain a good supply of infected cotton rats for chemotherapeutic investigations, during its progress, reports on various aspects of small-scale transmission have been published by Williams (1946), Scott (1946, 1947), Scott, Stembridge, and Sisley (1947). Hawking and Burroughs (1946), Bertram, Unsworth, and Gordon (1946), and Bertram (1946, 1947)

METHODS

The methods to be described were designed for transmitting Litomosoides to the maximum possible number of cotton rats with the minimum of labour. It is fairly easy to transmit this worm in the laboratory to a few animals at irregular intervals, but the maintenance of a constant supply of large numbers of infected animals is much more difficult, requiring constant care and attention to numerous small details. The chief difficulty is the prevention of foreign mites or insects entering the tanks which contain Liponyssus and rats, and destroying or overgrowing the Liponyssus. A second difficulty is encountered in the maintenance of proper conditions of humidity in the tanks, so that they are humid enough for the mites to

multiply but not so sodden with rat urine that the mites are drowned. The methods now employed have been subjected to repeated modification in the light of experience, and perhaps further improvements will still be found possible. Since success depends to such a large extent on the observance of small details, our present technique will be described rather minutely, after which the reasons for the various procedures will be discussed.

DESCRIPTION OF PRESENT TECHNIQUE

The process may be divided into five stages

- (1) The breeding of *Liponyssus bacoti* in pure culture
- (2) Infection of the mites with *Litomosoides* from infected rats
- (3) Infection of clean rats from the infected mites
- (4) Maintenance of the rats while the worms mature
 - (5) Examination of the infected rats

(1) Breeding of the mites in pure culture

The pure cultures are kept in the insectary described by Hunt and Davey (1947), in which the temperature is kept at approximately 24–26 C and the relative humidity at 75 to 85 per cent. This room is used primarily for the maintenance of adult mosquitoes, but it is convenient to use it also for breeding the pure cultures of mites as the atmospheric conditions are ideal, the cultures occupy little space and, furthermore, they are isolated from contamination by foreign mites which may occur in the room used for the next two stages.

The colonies of mites are maintained in small glass jars (1-lb jam jars) measuring approximately 8 by 13 cm Before use the jars are washed Clean sawdust about 2 cm deep is put in the bottom of each, and on top of this a small piece of clean white filter paper is placed The top of the jar is covered with finest bolting-silk or parachute nylon, the covering being tied round the neck with string. The jars and contents are sterilized in the autoclave (preferably the drying autoclave) and subsequently left 1-2 days in a 37° C incubator to become thoroughly dry The string fastening the silk covering is now removed from each jar and replaced by a rubber band Mites (adults only) are removed from existing cultures by means of a fine camel's-hair brush (previously cleaned by dipping for a few minutes in ether) and floated off on to the surface of some clean water to remove any adherent matter They are then transferred, again by brush, to a sheet of filter paper on which they are examined with a lens in order to make sure they are really L bacoti and that they have no smaller mites crawling on them, as is often the case After examination they are transferred to the filter paper inside

the jam jars A thin film of dimethylphthalate smeared round the lip of a jam jar will prevent the escape of mites when the silk covering is removed The effects of this repellent do not last more than one day, however Each day one, or if possible two, day old mice are put into each jam jar, or a day old rat can be inserted every second day. To prevent con tamination of the culture with unwanted species of mites which may be carried on the mice, each animal is placed in ether for about 10 seconds and then dried thoroughly in the air before being put into the jar The mouse should be held gently with forceps throughout this operation Mites feeding on a baby rat are shown in Fig 1 Dead mice are removed daily with forceps, adherent mites being shaken from them back into the jar Starting with ten mites in one par it is possible in this way to breed about five thousand mites in a month of which half may be expected to reach maturity

A quicker but less safe method of "seeding" individual jars with mites is to remove a mouse from a jar which is already crowded with mites and to put it into the new jar. As the mouse will have a number of mites feeding on it at any given time, it is thus possible to carry over as many as a hundred mites in a single operation. However, mites should always be handpicked into one or two jars in order to maintain the stock culture, since otherwise there is considerable danger of contamination with foreign mites.

(2) Infection of the mites with Litomosoides from infected rats

This is carried out in an animal room set aside for the purpose The temperature is kept between 27 and 32° C, it is never allowed to fall below 24 C, as this will prevent L bacoti breeding at the optimal The relative humidity is maintained at about 50 per cent or 80 per cent, according to circumstances (see below), by means of a bucket of water standing over a small electric fire, the position of the bucket relative to the fire is adjusted when required accord ing to the reading of a hygrometer on the wall This simple arrangement is quite satisfactory for the present purposes The mites (and rats) are kept in rectangu lar tanks, measuring 54×50 cm by 26 cm high, and made of 20-gauge galvanized iron sheeting (Fig 2) The joints are brazed and riveted and the top edges of the sides are rolled, 4 cm below the top edges, on the outside of each tank, is a horizontal ledge 17 cm wide with an outer wall 13 cm high, forming a channel which is normally filled with waste engine oil A rectangular wooden frame, of 36 cm depth and 52 cm wide, fits the top of each tank, on the under side there is a groove 1 cm deep to fit over the edge of the tank, the recess is packed lightly with cotton wool, which is soaked with liquid paraffin bolting-silk or parachute nylon is stretched over each frame and stuck down with petroleum jelly, and fur The joints of the ther secured by nailed laths wooden frame are packed with a plastic mixture of paraffin wax and petroleum jellv Some metal tanks

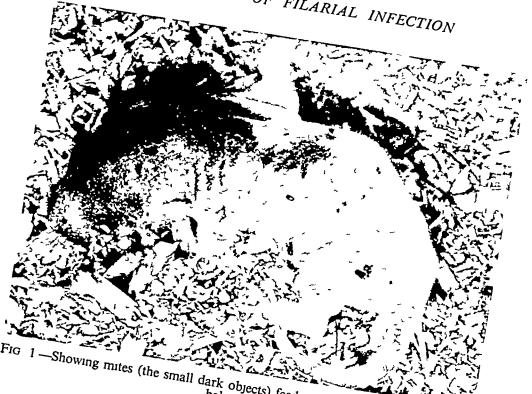


Fig 1—Showing mites (the small dark objects) feeding on the posterior part of a baby rat

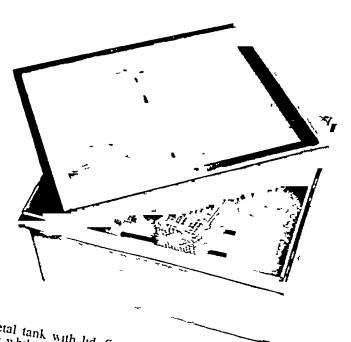


Fig 2.—Metal tank with lid (inverted) used for maintaining the colonies of mites while in contact with infected rats. Note (1) the groove in the lid packed with white cotton-wool to seal the top of the groove in standing on two pieces of wood, on blotting-paper, on sawdust.

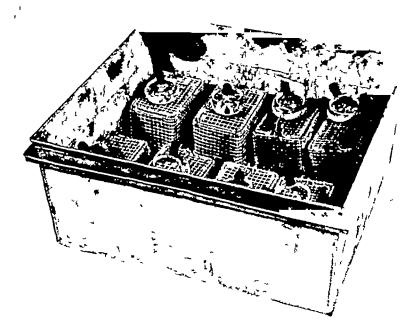


FIG 3—Metal tank containing eight clean rats in separate cages for exposure to infected mites, the glass sphere on top of each cage is a water bottle



Fig 4—Room used for storage of rats while infection develops. The cages are stood in double rows on metal bars, with a tray of sawdust underneath to catch the droppings

are also used of smaller dimensions viz., 50 cm > 40 cm by 15 cm high. The wooden lid of these is 10 cm deep, so as to compensate for the shallowness of the tank These second tinks do not have an external oil gutter. Consequently they must be stood on metal tables 125 × 60 cm by 75 cm high which have an oil putter 3 cm > 55 cm deep round the margin so as to prevent the escape of mites into the room. The tanks provided with in oil gutter are also stood on such isolated tables as a general rule although they can also be stood on in ordinary table 75 cm high. Wooden furniture is avoided as fir as possible in this room, so as to word infestition with bed bugs, if a wooden table is used the legs are stood in small basins of oil or lisol

Livery effort is made to ensure that each unit trink and lid) is proof igninst the entry of any small faunt -e g mites other than L bacoti, lice, flies-which can prove dangerous to the life of L bacoti. Nothing is put into the infection tanks which has not previously been sterilized or thoroughly elevated found useful to cover the hands and wrists with insect repellent (e.g. dimethylphthalate) before handling the tanks or their contents. Before use the tanks are steam sterilized. The lids are sprayed with xvlene to I'll any small fauna that may be present, they are placed on the tanks and allowed to dry sterilized in the drying autoclave is sprend to a depth of about 5 cm, over the bottom of each tank cotton rats infected with L. carena and showing microfiltrite in the peripheral blood are isolated in separate sterilized wire cases 15 cm long < 10 × 10 cm and sprayed with xylene from a scent-spray (Great care is needed in order to avoid killing the rats with the xylene aerosol) The cages are supported on clean metal rods resting on two opposite sides of a shallow tray containing dilute lysol and the rats are left there until all trace of xylene fumes has disappeared The cages, with enclosed rats, are then placed in the tanks containing the sawdust Four thicknesses of dry sterilized blotting-paper are placed under each cage to eatch excreta. The cage is raised about half a centimetre above the blotting-paper by being laid across two small sticks so that accumulated facces do not soil the bottom wires of the cage. The blottingpaper is changed every two days. Usually two infected rats are allotted to each tank, but more may become desirable if mites are very numerous Mites are added to the tank by pouring into it the contents of one or more of the jars containing mite-colonies described in the previous section

During their sojourn in the tanks the rats are kept on a minimum diet. Two to four grammes of cubed processed food, previously baked to sterilize the surface, and ten cubic centimetres of water per rat daily are sufficient. Particles of waste food must not be allowed to accumulate, and excess of urine must be avoided. Since the rats are serving to encourage breeding of the mites, as well as to infect them, they are left in the tanks for seven days, or until the mites are "swarming" (not only near the rats, but also over the whole of the sawdust), whichever time is the

greater (The mites show great activity if the observer breathes on to the sawdust). The rats are then removed from the tanks and are 'demited' passively by being left for a two-day period over the tray of lysol described above.

(3) Infection of clean rats from infected mites

The same tanks are used as in the previous section Within three days of taking out the infected rats, two or three cases measuring 20 × 28 cm by 10 cm high are placed in the tank. Each cage contains five young female rats which have been freed from foreign mites by means of xylene as described above. Alternatively eight of the small enges containing single rats may be inserted (Fig. 3). The blotting-paper is changed every dry except Sunday to prevent the accumulation of tirine and freces The soiled blotting-paper and the facces lying on it usually contain many mites, in order to allow these to fall back on to the cage, the paper and its adherent faeces are stored for one or two days on top of the cages inside the tank before they are thrown away The positions of the cages inside the tanks are transposed every day so as to compensate for any maldistribution of mites humidity of the room is adjusted according to the number of rats present. If few are in the tanks, the relative humidity is raised to 80 per cent, if many are present in the tanks, the humidity is allowed to fall to 50 per cent in order to encourage the drying of the blotting-paper and sawdust. A few days after the clean rats have been inserted, about 30-40 mites may be removed from the tank and dissected to look for worms. This confirms that the mites will become infective otherwise the further procedure must be modified accordingly

After fourteen days the clean rats are removed and demited as was described above. If the empty tanks seem well stocked with mites and free from contamination by foreign fauna, infected rats are inserted in order to infect the mites again, thus re-starting the whole cycle. Otherwise the contents of the tank are placed in an incinerator and the tank is cleaned and sterilized, after which it will be available for use as at the beginning

(4) Maintenance of the rats while the worms mature

After the rats have been removed from the infection tanks and demited, they must be stored for about fifty days while the worms mature. During this waiting period, female rats can be kept in cages of five, males must be kept in separate small cages (15×10×10 cm). All cages have wire bottoms. They are arranged across the longitudinal bars of a big metal rack (Fig. 4). A few centimetres below each row of cages there are long metal trays to catch the excreta. The trays contain sawdust and are changed every two days. The rats are provided with an excess of the Institute cubed stock diet for rats and mice, supplemented by green cabbage three times weekly. Water is provided in excess in licking-bottles. The temperature of the room is maintained at approximately 25° C.

(5) Examination of the infected rats

Cotton rats are more difficult to handle than ordinary laboratory rats, since they are easily frightened and try to bite when gripped, also the skin on the tail is too fragile for them to be lifted up by this We have found that the most satisfactory technique is to approach the rat suddenly from behind and grasp it firmly by the scruff of the neck worker with sufficient confidence in himself can do this with the bare hand, without risk of injury, others will require stout leather gloves to give them a feeling of safety An alternative technique is to take hold of the body by the hand protected by a leather glove so that the head projects between the thumb and fore-The rat twists round to bite the glove near the base of the thumb, but with a suitable glove there is so much slack leather at this point that the teeth do no harm Meanwhile, blood can be easily taken from the tail or injections can be made into the posterior half of the animal If there is difficulty in extracting a rat from a small cage, the rat is emptied out into a small dustbin more than 80 cm deep-1 e, too deep for the rat to jump out In this it can usually be grasped easily

About eight weeks after removal from the infection tanks a drop of blood is taken from the tail of each rat and smeared on a slide to make a thick film about 2 sq cm in area. These films can be stained by any of the usual techniques for microfilariae. We have found it rapid and convenient to dry them, to dehaemoglobinize in 15 per cent acetic acid, to dry again, and to stain with Leishman's stain.

After examination each rat is kept in a separate small cage. Rats which fail to show microfilariae three months after removal from the infection tanks are considered not to be infected, and they can be exposed to infection once again. We have no reason to believe that such rats are protected by a natural immunity, but the matter requires further investigation. If the number of infected mites in the tanks is high, there should be few failures to infect rats.

Comments on the Technique for Obtaining Rats Infected with *Litomosoides*

The previous section has described, somewhat dogmatically, the technique at present in use Various features of this technique may now be discussed in more detail, to show the reasons for adopting them and our experience with alternative procedures

(1) Breeding of mites in pure culture

The technique (described above) for breeding mites in jam jars has been in use for a year. It was started at a time when the mite population at our disposal was dangerously low, and the first jars contained soil and hay in imitation of the

nat infection boxes then in use Our whole present population is derived from three jars of one dozen mites each. We have not once had a jar contaminated with foreign mites. It is considered that this technique is more convenient and safer from contamination than the Single Unit technique described by Bertram, Unsworth, and Gordon (1946) or than methods requiring the isolation of mites after every feed

Some observations on the habits of these mites are made possible by this technique. If the mites are well fed, with an excess of hosts (mice) present, they will not be seen above the sawdust except when actually feeding' Examination of the saw dust with a lens will reveal numerous stationary adults and highly active unfed protonymphs making their way upwards Where the mites have insufficient food, however, they run rapidly over the inside of the jar, and will congregate on the inner side of the silk covering if there are mammals in the room As soon as a mouse is put into the jar, further large numbers of mites will emerge from the sawdust and run rapidly about in the vicinity of the mouse They appear to be under the influ ence of a simple chemotaxis which causes them to move directly towards the source of stimulation, until within one or two centimetres of it, when they will skirt about for some minutes before arriving at the source itself Once on the mouse, young protonymphs will begin to feed almost at once The older mites will run over the skin for as long as half an hour, before taking up a position to feed, usually at an orifice or in a fold of the Behind the ears, in the groin, in the fold of skin between the shoulders, and around the mouth and nostrils are the favourite feeding The mites appear to stop running when they touch a feeding mite, and not infrequently start feeding themselves In this way little clusters of mites soon form (Fig 1) The older mites do not seem to interrupt their feeding as frequently as the protonymphs do

(2) Infection of the mites from infected rats

As was said above, the maintenance of healthy colonies of mites during this and the next operation depends chiefly on the avoidance of contamination by other small fauna and the maintenance of appropriate humidity in the bedding The tanks used were at first uncovered, but contamination with foreign mites persistently developed in spite of all precautions to prevent the accumulation of waste food and faeces Even with the covers, most tanks become contaminated within six weeks of setting up When contamination occurs, the

contents of such tanks should be sterilized as soon as the cycle of operations can be profitably terminated since the population of *Liponyssus* usually diminishes rapidly, and the tank acts as a source of contamination for other tanks

The commonest contaminants are mites of genera other than Liponyssus. When silk covers for the tanks were not used, the forage mite Tyrophagus castellanu Hirst (and probably other similar species) was most frequently found swarming over the frarments of food dropped by These mites the rats, and on the licking-bottles had no appreciable effect on the I bacon. Less frequently a large vellow mite (adults 10 to 15 mm long) Macrocheles carmatus Koch, would appear, and its appearance always heralded a decrease in the numbers of L bacoti so that we were led to the conclusion that this mite would eat either More recently a smaller L bacon or its eggs vellow mite (adults 0.5 to 1.0 mm, long). Hypoaspis freemant, has been a frequent visitor, with similar disastrous consequences for L bacati. We have had little trouble from the Insecta | Lice have been seen once or twice presumably feeding on the rats, but never in great numbers and never to the detri-A Dipteran Coprophila ment of L bacon ragans Hal (a small black fly superficially resembling Drosophila) troubled us before the silk covers were introduced. It bred among the rat faeces and probably carried other contaminants from one tank to another

The blotting paper underneath the cages was introduced in order to catch the urine, faeces, and waste food from the rats and to reduce soiling of the bedding Pieces of cardboard were first employed and were moderately satisfactory, but they were difficult to clean and too expensive to ienew every two days. Metal plates were tried but they collected pools of urine, in which the mites drowned so they were quite unsuitable Initially the waste material from the cages matted together in the meshes of the wire floor and formed an impervious sheet, this was obviated by inserting a small wooden bar, about 0.5 cm, thick, between the blotting paper and each end of the The blotting paper should not project more than about 1 cm round each cage, lest it hinder passage of the mites between the cage and the underlying sawdust Young rats tend to gnaw the paper through the floor of the cage, reducing the upper surface to a powdery mass, this does little harm, apart from the mechanical inconvenience The supply of drinking water to the rats has to be adjusted so as to prevent the blotting paper becoming soaked with excess of urine-otherwise conditions become unfavourable for the mites

amount of urine which can be tolerated depends on the humidity of the atmosphere, on the number and size of rats in the cage and on the frequency of changing the paper

The bedding used in these cages originally consisted of a layer of dry sterile earth covered with sterile hav or straw as described by Williams (1946) Sawdust was found to give equally satisfactory results, with the additional advantages of cleanliness and the provision of a good background against which mites could be easily seen. The rats used to infect the mites should contain numerous nucrofilariae the blood of our donors usually contains more than 50 microfilariae per cu mm If the mites are too numerous they may cause death of the rats by exangumation, the dangerlimit can be recognized only by experience. When it is approached, the donor rats can be changed more donor rats can be added, or as a last resort the number of mites may be reduced by judicious spraying with Sylene

The percentage of mites which develop filarial infection during our procedure is small. Sample dissections have been done at various periods after exposing rats to the mites for 7-14 days. In 716 mites dissected within 6 days of the last day of exposure, 4.3 per cent were infected, of 419 mites dissected between the 7th and 13th days after exposure 1.4 per cent were infected, while of 240 mites dissected 14 or more days after exposure, none were found infected. For various reasons, these percentages are probably lower than the actual rates among the mites.

The length of the worms found in these mites varied from $60~\mu$ to $800~\mu$, according to the period which had elapsed since the worm was ingested by the mite. This infection rate of the mites is much lower than has been reported by some observers—e.g., Bertram (1947) speaks of infection rates of 38 per cent. In our colonies the mites which have fed on the infected rats are constantly being diluted by young mites which have not so fed. All the same, there are sufficient infected mites in the colonies to transmit the parasite readily, and even in tanks where 50 or 100 mites were dissected without finding worms 50–100 per cent of the exposed rats became infected

(3) Infection of clean rats from infected mites

The general problems of this stage are the same as those of the preceding stage. Greater, care is required to prevent the blotting paper and bedding getting too wet. The caging of the cotton rats during this and the subsequent stage depends on their age and sex. The rats are most conveniently kept in groups of five, since each rat has more

freedom and the whole group occupies less space than five separate small cages. Unfortunately, confined groups of adult male rats or of mixed sexes are usually decimated by savage fighting, so that when pubescent or mature males are used they must be kept in separate small cages (Fig 3) Restriction of use to female rats is convenient for handling, but it is rather wasteful of the breeding facilities

(4) Maintenance of rats while the worms mature

The chief difficulties encountered during this phase arise from the number of rats involved and the problem of intercurrent mortality dozen rats are removed from the infection tanks weekly and each must be stored eight weeks. accommodation is required for about two hundred rats, many of them in single small cages arrangement described (Fig 4) is the most compact, most convenient, and most economical of - attendant labour that we have vet been able to At various periods there have been many deaths among our rats, sometimes averaging two or three per day, so that a quarter of our rats were being lost in this way before the infection matured The causes of these deaths could not be satisfactorily determined The distribution on the animal racks was not suggestive of contagious spread Post-mortem examination of dead rats showed reddened intestines in some, thrombosis in the left auricular appendage of the heart in others, while others showed a clear effusion in the pleural cavities, intranasal instillation of this effusion into two other rats caused no obvious symptoms gical sections from a few of the rats suggested an infection with S typhi-murium Feeding-experiments showed that growth was improved by adding green food to the diet, but otherwise the stock cubed diet for rats of the Institute seemed adequate It was concluded that such sporadic deaths were best combated by attention to the general hygiene and diet

(5) Checking the rats for the presence of microfilariae in the peripheral blood

Microfilariae do not appear in the tail blood of infected rats until 42 days after their removal from the infection tanks (We habitually examine the rats for the first time after this period) This means a possible maximum of 56 days from the first exposure to infection, and agrees with the conclusions of other workers that 51–54 days is the time required for microfilariae to appear With light infections the appearance of microfilariae

may be delayed until the 55th day after removal from the infection tanks. This may mean that therats were not bitten-by infected mites until their last day in the tanks, or that the number of microfilariae in the blood was too low to show in the first blood samples

With most of our rats counts of microfilariae taken between the 40th and 60th days show a fairly consistent density of 10 to 15 per cubic millimetre of tail blood (about 2 per microscope field (\times 60) of an average thick smear), thereafter the count increases to between 100 and 300 per cubic millimetre at the 100th day, and it remains nearly constant after that (so long as the adult worms are healthy)

ADULT L Carinii in the Host

We have never found adult worms in any part of the host other than the pleural cavity (and occasionally the mediastinum) However, we have occasionally failed to find worms in their normal location, although the blood of the host was positive for microfilariae, and so the possibility of successful development of the worms outside the thorax cannot be excluded

Healthy worms in a healthy host tend to spread thinly over the wall of the pleural cavity, adhering by capillarity to the moist surface They are seen to wriggle continuously in the dissected host, perhaps they are less active if undisturbed If there is fluid in the cavity, the worms sink under the influence of gravity and become matted together They also become matted if subjected to unfavourable conditions, e.g., if the host is treated with antifilarial drugs When killed by drugs the mat of worms is soon covered with a fibrous deposit In rats which have been infected for more than three months we have occasionally found spontaneous cures Here, as in the drugged rats, we have found the worms matted together and covered with fibrin In order to avoid mistaking such spontaneous cures for the results of drug action therapeutic tests are best performed with newly infected rats

The worms are best examined in their living state. After removal from the pleural cavity the worms should be immersed in warm Ringer's solution and put in a 37° C incubator for half an hour. At the end of this time normal worms show active motility, particularly in a bright light. They are nearly transparent, and most of their internal structure can be made out with care. We have noticed that after treatment with drugs of varying degrees of potency the behaviour and appearance of the worms are modified in a fairly constant fashion. The weakest effective drugs (or doses)

induce sluggishness, while stronger ones result in reduced transparency of the worms' tissues. When the worms are actually dying as a result of therapy they become covered with a thin layer of phagocytes, and later with fibrin and they acquire a brown coloration. Even at this stage they may retain some motility. These changes are best seen a week after the course of injections has terminated and they may be absent in rats killed earlier than this

A moderate number of worms in the pleural cavity does not induce any gross pathological changes. If the number is excessive, however, the surface of the lung becomes inflamed and purulent. This may be due, to penetration of the tissue by microfilariae.

Spontaneous death of the worms has never been found to occur in less than three months after removal of the rats from the infection tanks. When it does occur, the dead worms are usually found in a dense capsule of fibrin, and they exhibit little or none of their normal structure.

Transmission of Litomosoides to animals other than cotton rats

In the early descriptions of the worm now called L carmin it was reported in various other small rodents, viz, Sciurus, and Hesperonius (Travassos, 1919), Holochilus vulpinus (Mazza, 1928), and Nectonius squamipes (Vaz, 1934). Accordingly, an investigation was made of the possibility of using other small rodents as experimental hosts for Litomosoides in place of the cotton rat. Animals were exposed to infection by the technique described above and the results are summarized in Table I. A preliminary note on these findings was published by Hawking and Burroughs (1946).

TABLE I
SUMMARY OF ATTEMPTS TO TRANSMIT Litomosoides
TO ANIMALS OTHER THAN COTTON RATS

White mouse Guinea-pig Hamster (Cricetus auratus)	Number of animals							
	Exposed	With worms in pleura	With Mf in blood or pleura					
Piebald rat White mouse Guinea-pig Hamster (Cricetus auratus) Orkney vole (Microtus orcadensis)	23 70 2 3	5 11 0 3	3 2 0 1					

The piebald rats and mice which did become infected were consecutive ones out of a series spread over many months, this suggests that if the others had been exposed to mites as heavily infected, they also would have developed worms Some of the animals contained up to thirty worms, but most of them contained less than ten, both of which numbers are low for cotton rats. In piebald rats the microfilaria count in the peripheral blood rose to 1,000-5,000 per c cm, and in two of the rats they persisted for more than two and three months respectively, the rats were then killed. In all the animals of Table I the infections were much lighter than those usually found in cotton rats and there seemed to be a tendency towards spontaneous cure It was concluded that although L carini will obviously develop in quite a variety of rodents (as shown by the literature and our own results) none of the species studied formed an efficient substitute for the cotton rat, which in fact remains the only satisfactory animal for chemotherapeutic experiments on this worm

CHEMOTHERAPEUTIC TESTS ON INFECTED RATS

In designing a chemotherapeutic test consideration must be given to (1) allowing the drug sufficient opportunity to show its antifilarial activity (if any), (2) avoiding undue consumption of laboratory time, labour, and material Culbertson and Rose (1944b) have shown that the effect of treatment of L carini infections with antimonial compounds must be judged by examination of the adult worms, since the microfilariae of the peripheral blood are not affected by doses which kill the adults On the other hand, Hewitt and his colleagues (1947) have recently reported that piperazine derivatives act mainly on the microfilariae and only to a much smaller extent on the adult worms Accordingly. examination of both adults and microfilariae is necessary in judging the antifilarial action of an unknown compound Our preliminary experiments with neostam (Table II) suggested that treatment given on six successive days was more effective than treatment on three days only, but treatment on twelve or eighteen days (which used much more time, labour, and drug) was not obviously more effective than treatment on six days In view of these results and of reports by American workers, it was decided to utilize a dosage schedule of six daily doses The procedure adopted is as follows

When a compound is to be tested, a rough estimate is first made of its chronic toxicity for mice when given by intraperitoneal injection once daily for four successive days. Two mice are used per dose and each dose is approximately twice the

TABLE II
SPECIMEN RESULTS OBTAINED IN TESTING COMPOUNDS FOR FILARICIDAL ACTIVITY

	Max tolerated daily dose for mice		Treatmen	t of rats		
Compound	(chronic toxicity) mg per kg	Dose mg per kg	No of doses	Interval (days)	No of rats	Condition of adult worms*
Neostam (stibamine glucoside)	250	40 40 80 80 130 130 130 130 160 250 260	6 18 6 12 3 3 6 12	1 1 2 1 1 1 3 -	2 2 1 2 2 2 2 2 2 2 1 2	Majority alive, a few dead All alive All alive All alive All alive in one case, all dead in the other All dead in one, majority dead in the other All dead in one, majority dead in the other All dead All dead All dead All dead All dead All dead All dead All dead
Pentostam (sodium stibogluconate)	2,500 _	500 1,300	6 6 ` 6	1 1 1	1 1 1	Females injured, males un affected All dead except a few of the males All dead
Anthiomaline	100	45	6	1	2	Males unaffected, females dead
Tryparsamide	2,500 -	250 500	6	1	2 2 1	Majority alive, a few females dead Males alive, females dead or dying
Neoarsphenamine	250	100	6	1	2	All dead
p-Methylsulphonyl benzamidine hydrochloride (V 187)	250	20–50	6	1	3	All alive

^{*} Microfilariae were unaffected in all experiments

one below it If the highest dose which has not killed any of the mice is x mg per kg, the dose used for the therapeutic trial in cotton rats is 0.4x mg per kg, given by intraperitoneal injection for six successive days, this dosage seldom proves toxic for the cotton rats. If the compound is found to be active, its toxicity can be investigated more closely. Infected rats are taken and a rough estimation is made of the number of microfilariae in the blood by counting the larvae in several typical microscope fields of a thick blood film. The rats are then treated by intraperitoneal injection given once daily on six successive days. Gotton rats infected with L carmu do not tolerate the in-

traperationeal injection of drug quite so well as healthy rats do Also cotton rats are more susceptible than other laboratory animals to sepsis and bacterial infections during this procedure. All drugs should be dissolved in sterile fluid, and kept in sterile containers. Syringes and needles should be sterilized before use, and the site of the injection should be well cleaned with cotton-wool soaked in ether or in dilute alcoholic iodine solution before performing the injection.

Six days after the last dose the blood is examined again for microfilariae, the rat is killed, its thorax is opened, and the worms found in the pleural cavity are removed and examined. If their

moulity is not obvious they are suspended in Ringer's solution and warmed to 37 'C, which often stimulates them to motility. Often a mass of worms is found and it mis be necessity to examine them under a microscope in order to estimate the proportion of living and dead one After effective filaricidal treatment, eg, large doses of antimonials, the worms, ire usually found embedded in fibrin forming small compact white The microfilmae are much more rea tant to treatment (by compounds other than piperazines) than the adult worms are and when the adults have been killed by antimonials many active microfilariae can be found in the ptem d fluid of the rat, living microfilative also persit in the peripheral blood, where we have found them in gradually diminishing numbers for more than three months (as described by Culberrion and Rose 1944b)

Examples of some of the therapeutic tests are reproduced in Tible II. As reported by other observers adult worms can be falled by suitable doses of antimonials (e.g. neostam, pentostam or anthiomaline) and of arsenicals (e.g. ti-pats amide). The males usually seem more resist not than the females. The microfilative are unaffected by these types of compound. Most of the substances examined to date have unfortunately been inactive e.g. V 187

DISCL SSION

In 1940 one of the authors (Hawking) described unsuccessful therapeutic investigations on patients with filariasis in East Africa and pointed out that although a chemotherapeutic remedy for filariasis would eventually be found, the testing of compounds directly on human beings was unlikely to produce the desired solution on account of the many difficulties and limitations inherent in such work, in order to be able to seck such a remeds with good prospects of success it was necessity first to obtain "an animal carrying a filarial infestation suitable for laboratory experiment, thus the animal must be small and cheap and the problem of transmitting the infestation, presumably by insect vector, must also be solved." The infestation of cotton rats with Litomosoides described above, seems to fulfil the conditions then laid down, and the prophecy then made that when this postulated experimental animal was available "the discovery of a potent filaricidal substance would be only a matter of time" is apparently in the process of fulfilment by the piperazine derivatives reported by Hewitt and his colleagues (1947) The filarial infections hitherto available in the 50, 85

for the photography, and to Miss P Davey and Mr D Garlick for technical assistance

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SUPPLEMENTARY NOTE

Since this text was written the following modification of technique has been introduced. Blocks of plaster-of-Paris 2 0-2 5 cm thick are used to absorburine and collect faeces in the infecting tanks, they take the place of the blotting-paper mentioned on p 289. Each block is of such a size as to accommodate four small cages or one large cage with 1-2 cm overlap. Faeces are periodically scraped off each block into a perforated zinc tray which hangs from the side of the tank, mites, which are usually plentiful in the

faeces, can then return to the sawdust. Between periods of service in the tanks, which may be of up to two months' duration, the plaster blocks are steeped in water and then dried, when they are ready for use

An important paper (Williams, R W, 1948, J Parasit, 34, 24) entitled "Studies on the life cycle of Litomosoides carini" appeared early this year, too late to be discussed in the text

PHENYLTHIOURETHANES AS LOCAL ANAESTHETICS

BY

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Huang Lu, and Chang (1946) found that the hydrochlorides of sym bis(diethylamino)isopropyl phenylurethane and sym dipiperidino-isopropyl phenylurethane exhibited marked local anaesthetic potency and relatively low toxicity. In view of these results and the similarity between the chemical behaviour of oxigen and sulphur, the hydrochlorides of two new phenylthiourethanes viz diethylaminoethyl phenylthiourethane (SI) and sym dipiperidino-isopropyl phenylthiourethane (SPP), have been prepared and tested for toxicity and local anaesthetic activity. The formulae of these two compounds are given below

(SD) C₁H₂NH CS OCH CH₂N(C₁H₂)₁,HCl (SPP) C₁H₂NH CS OCH(CH₂NC H₃) 2HCl

EXPERIMENTAL

Diethylaminocthyl phenylthiourethane hydrochloride was made by allowing sodium diethylaminocthoxide (prepared from diethylaminocthoxide (prepared from diethylaminocthoxide (prepared from diethylaminocthoxide (2 35 g) and powdered sodium (0 45 g) in xylene (6 cc) on a steam-bath) to stand overnight with phenyl isothiocyanate (3 g.) The product was acidified and the xylene removed in ether. The free base was an oil, but the hydrochloride crystallized from ethanol-ether, mp 121-122° C, yield, 43 g (Found C, 540, H, 74, N, 100 C₁₃H₂₁OSN_Cl requires C, 540 H, 73, N, 97 per cent)

Sym dipiperidino-isopropyl phenylthiourethane was prepared similarly from dipiperidino-isopropanol and phenyl isothiocyanate. The free base crystallized from 90 per cent alcohol, mp 125-126° C (Found C, 666, H, 86, N, 117 C₂₀H₃₁OSN, requires C, 665, H, 87, N, 116 per cent.) The hydrochloride crystallized when the free base was dissolved in warm 3N hydrochloric acid, needles, turning red at 200°, mp 235-236° C (Found N, 100 C₂₀H₂₁OSN₂Cl₂ requires N, 97 per cent.)

Toxicity and anaesthetic potency

The experimental methods used were similar to those described previously (Huang et al, 1946) except that guinea-pigs were used instead of human subjects for the intradermal weal tests. The results are collected in the Table. It will be seen that SD was about two-thirds as toxic as cocaine and SPP about

TABLE TONICITIES AND ANAISTHETIC POTENCIES

Drug (Karber		Annesthetic potency (effi- ciency ratio, average of 7 experiments)				
	method)	Rabbit s cornea	Intradermal weal (guinea-pig)			
SD SPP Cocune Procune	165 (50)† 97 (50)† 102 (50)†	213 5	13 1			

t Total number of mice in parentheses

equal in toxicity to cocaine. SD was about two thirds and SPP about five times as potent as cocaine when tested on the rabbit cornea. When compared with procaine by the intradermal weal method, SD and SPP were about two and thirteen times as potent respectively.

The practical value of a local anaesthetic depends among other things upon its being non-irritant and stable to sterilization by heat. It was observed that both SD and SPP produced congestion of the conjunctiva in the rabbit's cornea tests and necrosis of the skin in the intradermal weal tests in concentrations at which local anaesthesia was achieved

When solutions of SPP and SD were sterilized at 100° C for one hour, the anaesthetic activity of the former was completely lost but that of the latter remained unchanged

SUMMARY

The hydrochlorides of diethylaminoethyl phenylthiourethane and sym dipiperidino-iso-propyl phenylthiourethane have been synthesized Both exhibited marked local anaesthetic activity in the rabbit cornea and intradermal weal (guineapig) tests, but they are regarded as unsuitable for clinical application because they were found to be irritant. They had toxicities in mice of the same order as cocaine.

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CURARE-LIKE ACTION OF POLYMETHYLENE BIS-QUATERNARY AMMONIUM SALTS

ΒY

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(Received May 11 1948)

Tubocurarine chloride was shown by King (1935, 1936) to be a bis-tetrahydroisoguinolinium salt containing two quaternary nitrogen atoms. The curare-like blocking of neuromuscular transmission is a property of "onium cations" in general, and it was thought that the exceptional potency of tubocurarine compared with simple quaternary ammonium ions might be due in part to the presence of two such cationic groups at some optimal distance apart in the same molecule With this in mind we have prepared and tested for curare-like activity a number of simple bisquaternary ammonium salts in which the nitrogen atoms were directly attached to the terminal carbon atoms of polymethylene chains of different Such salts may be conveniently referred to as 'bis-onium salts", they were all polymethy-lene bis-onium dibromides of the general type

$$Br\left\{\begin{array}{c} N-(CH_2)_n-N \end{array}\right\}Br$$

Few compounds of this type have been examined previously, Brieger (1886) observed that tetramethylene bis-trimethylammonium hydroxide had some curare-like action in intact animals, and Ackermann (1921) made similar observations with the analogous ethylene and pentamethylene bis-trimethylammonium aurichlorides

The following salts have been investigated

- 1 Bis-trimethylammonium dibromides, $Br\{Me_3N(CH_2)_nNMe_3\}Br$, where n was 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, and 13 They are referred to as BTM followed by the number (n) of methylene groups in the chain, thus trimethylene bis-trimethylammonium dibromide is referred to as BTM3
- 2 Bis-triethylammonium dibromides, Br{Et₃N(CH₂)_nNEt₃}Br, where n was 2, 3, 4, 5, 7, 8, 9, 10, and 13 They are referred to as BTEn

- 3 Bis-strychninium dibromides, Br $\{C_{21}H_{22}O_2N_2(CH_2)_nC_{21}H_{22}O_2N_2\}$ Br, where n was 2, 3, and 5 They are referred to as BSn
- 4 Bis-quinolinium dibromides, Br $\{C_pH-N(CH_2)_nC_pH-N\}$ Br, where n was 3, 5, and 10 They are referred to as BOn
- 5 Bis-(phenyldimethylammonium) dibromides, Br{PhMe₂N(CH₂)_nNMe₂Ph}Br, where *n* was 3 and 5 They are referred to as BPDM*n*

The following abbreviations will also be used TM for tetramethylammonium iodide and TE for tetra-ethylammonium iodide

-A brief note on some of the results with these bis-onium salts has already appeared (Barlow and Ing. 1948) -

МЕТНОВ

The choice of a method for comparing the curare like activity of numerous compounds is a matter of some difficulty; older methods have been reviewed. by Ing (1936) Recently two methods have been devised for assaying tubocurarine solutions Holaday (quoted by Bennett, 1941) developed the rabbit head drop method and Chou (1947) used the isolated phrenic nerve-diaphragm preparation of the rat We have tried to adapt Chous method to the comparison of the curare-like activities of our bis-onium salts Initially the apparatus and procedure were exactly the same as those used by Chou, except that the bath was smaller (50 ml instead of 100 ml) denser discharge stimuli used by Chou were of about 10σ duration and as there was a tendency for the nerve to discharge more than once they were later replaced by square wave stimuli of about 010 duration It was also observed during the course of the work that the diaphragm preparation is very sensitive to small changes of temperature and careful control of the bath temperature is necessary

Chou allowed his tubocurarine chloride solutions to act for three minutes we retained this feature of the method because if the drug is allowed to produce its full effect so much time is occupied by the poisoning

active as TM (Ing and Wright, 1931) In the BTM series the least active contain polymethylene chains of 3, 4, and 5 carbon atoms (the hexamethylene member was not prepared), members with polymethylene chains of 7 to 13 carbon atoms are all much more active. However, it is doubtful whether it is legitimate to compare activities on a rat preparation with those on frog preparations.

In the rabbit head-drop test the members of this series displayed entirely different orders of activity, as will be seen in Table II activity rose to a pronounced maximum for BTM10, which was some three times as potent, weight for weight, as d-tubocurarine chloride and about 10 times as active as the salts with 8, 11, 12, and 13 carbon atoms in the polymethylene chain Only BTM9 approached BTM10 in activity, being about a third as active

TABLE II

COMPARISON OF BTM SERIES WITH TUBOCURARINE
CHLORIDE IN RABBIT HEAD-DROP TEST

Salt	Mean head-drop dose ± S E μg/kg		Relative potency
Tubocurarine chloride	261 ± 14	5	1
BTM8	808 ± 52	2	03
,, 9	290 ± 27	4	09
,, 10	78 ± 20	6	33
,, 11	657 ± 107	2	04
,, 12	783 ± 81	2	03
,, 13	857 ± 43	2	03

Bis-triethylammonium (BTE) series —The behaviour of members of this series had some resemblance to that of alkyltriethylammonium salts on frog muscle Kulz (1922) recorded a steady increase of activity in the series Et.N C.H.2n+1 from n=2 to n=8, and we have observed (on the rat diaphragm) a steady rise in activity from BTE4 to BTE13 The latter was about a hundred times as active as BTE4 and somewhat more active than In the rabbit head-drop test BTE13, BTM9 though the most active of the series on the rat diaphragm, was less active than BTM9 and about two-fifths as active as tubocurarine BTE2 and BTE3 were only feebly active, BTE2 resembled TE in augmenting the response of the muscle to stimuli which had previously given maximal contractions (see below)

Bis-strychninium (BS) series—The rat diaphragm is less sensitive to methylstrychninium salts than the frog's sartorius, thus, whereas Cowan and Ing (1934) found that methylstrychninium iodide was as active as tubo-

curarine chloride* on the isolated frog's sartorius (R esculenta), we found that the chloride was not more active than TM on the rat diaphragm. The rat diaphragm is more sensitive to TM_than the frog's sartorius thus, 0.25 mM solutions of TM produced 50 per cent inhibition in 3 min, whereas Ing and Wright (1931, Fig. 1) found that 2.0 mM solutions were required to produce a similar inhibition of the isolated frog's sartorius in a similar time. At the same time TM is much less potent than tubocurarine on the rat diaphragm.

BS2 was more than twice as active as methylstrychninium chloride on the rat diaphragm (com pare BTM2 and TM), but BS3 and BS5 were not significantly less active than BS2, a result which contrasts strongly with the feeble activities of the corresponding members of the BTM series All bis-strychninium salts produced augmentation of the contractions of the rat diaphragm before inhibition

Bis-quinolinium (BQ) series —BQ3 and BQ5, unlike the corresponding members of the BTM series, show moderate activity BQ10 was much less active than was expected, it was only about as active as BQ5, but it had a powerful augmenting effect on the muscle contractions, which must compete with the inhibitory effect.

Bis (phenyldimethylammonium) (BPDM) series—Only two, BPDM3 and BPDM5, were prepared and tested, and, like the corresponding BQ compounds, they were both moderately active

Augmentation effect —It has already been noted that some bis-onium salts increased the size of the contractions of the rat diaphragm to maximal stimuli With some salts, eg, BTE2, this was the main effect and inhibition only occurred after a lapse of time much longer than 3 min, with most salts the augmentation was only observed In the BTE series, apart in some preparations from BTE2, which invariably showed it, the augmentor effect was observed from time to time with all the other members except BTE13, it was less frequently observed in the BTM series preparations appeared to be much more sensitive to this augmentation (or possibly much less sensi tive to the inhibitory effect) than others, and this variability in the response of preparations to these two opposing effects was probably the main reason for the difficulty in obtaining consistent estimates of relative activities, moreover, there was rarely a clear differentiation between concentrations which produced mainly augmentation and those

^{*} The specimens used were King's crystalline d-tubocurarine chloride at that time recently isolated and Bochm's original amorphous alkaloid from tubocurare, they were wrongly referred to as curarine chlorides

optimal distance apart, we should expect high curare-like activity to occur with bis-onium ions capable of a similar separation of the two nitrogen atoms, such separation would probably involve a chain of at least 8 atoms. It is noteworthy that of the highly active bis-onium salts of types II and III studied by Bovet et al. (1946, 1947) the most active have 8 to 12 atoms between the two nitrogen atoms.

A reason may also be tentatively suggested for the occurrence of high activity in compounds containing two quaternary nitrogen atoms if, in virtue of the distance between them, both charged centres are held by the "receptive substance," simultaneous dissociation of both centres from the receptors will be highly improbable and, so long as one centre is held, the re-attachment of the second is made more probable owing to the proximity of the ion to the receptive substance

Our results with the BTM series indicate that the sensitivity of the rat diaphragm to bis-onium salts differs greatly from that of the rabbit observed that the rat diaphragm was less sensitive to methylstrychninium and more sensitive to TM than the frog's sartorius (R esculenta) Paton and Zaimis (1948) have noted that, compared with tubocurarine, BTM8 is some nine times as active in the cat as in the rabbit, and Brown and Dias (1948) have reported a striking difference in the sensitivities of Leptodactylus ocellatus and Rana temporaria to tubocurarine chloride These differences in species sensitivity suggest that synthetic curare-like-drugs ought to be tested on a variety of species

The inhibition of cholinesterases by some of our bis-onium salts was unexpected and the specificity of this effect would probably repay further investigation. There is a rough correlation between the intensity of this effect and that of the augmentor effect in the rat diaphragm, but other possible explanations of the latter effect have not been explored.

CHEMICAL SECTION

The bis onium dibromides were prepared by heating polymethylene dibromides in ethanol with a large excess of the requisite tertiary base for from 5 to 24 hours. In the BTM series 33 per cent (w/v) aqueous trimethylamine was used a pressure bottle was unnecessary.

Ethylene bis-onium dibromides were less easily prepared BQ2 and BPDM2 could not be isolated. The product of heating ethylene dibromide with strychnine in ethanol is bromoethyl-strychninum bromide (Found N, 5 62 C₂₃H₆O N-Br- requires N, 5 38%) BS2 was prepared by heating this half-way product with excess strychnine at 180° C for 7 hours in

glycerol as solvent, the glycerol was removed, after acetylation, as triacetin by ether extraction, excess strychnine was extracted with chloroform, yield almost theoretical

Condensations of polymethylene dibromides with quinoline and dimethylaniline give red and blue by-products respectively. The formation of red products with quinoline may be partly, and of blue products with dimethylaniline completely, suppressed by carrying out the reaction in an inert atmosphere (N, or CO₂), coloured products are best purified by pouring the reaction solution through a charcoal column

Bis-onium dibromides are white crystalline solids They are often both hydrated and hygroscopic, their analysis has presented some difficulty. Some salts could not be dried to a constant water content and the anhydrous salts were too hygroscopic for successful analysis, it was necessary to analyse the hydrated salt and use the experimentally determined water content in calculating the theoretical carbon and hydrogen When an attempt was made to analyse some anhydrous salts, in particular BTE11 and BTE13, the specimens absorbed appreciable amounts of water between weighing and combustion, this can be detected by the high value for hydrogen and the low value for carbon, and if the excess hydrogen is assumed to be derived from water a 'corrected' value for carbon can be obtained

Analyses are by Drs Weiler and Strauss All mps are uncorrected

Ethylene bis-trimethylammonium dibromide BTM2
Crystallized from ethanol No mp below 280°
Found C, 315, H, 79 C₈H. N₂Br₂ required
C, 314, H, 72%

Trimethylene bis-trimethylammonium dibromide BTM3
Crystallized from ethanol Loses water above 105°
but does not melt below 250° Roth (1881) does not record a mp

Found C, 32 5, H, 7 87 Calculated for C₉H₂₄N₂Br₂,1H₂O C, 32 0, H, 7 70%

Tetramethylene bis-trimethylammonium dibromide BTM4
Crystallized from ethanol Loses water below 110° but
does not melt below 305° v Braun and Lemke (1922)
recorded mp 295° (dec)

Found C, 327, H, 780 Calculated for C₁₀H₂N₂Br, 2H₂O C, 324, H 811%

Pentamethylene bis-trimethylammonium dibromide BTM5 Crystallized from ethanol No m p below 280° Found C, 37 2, H, 7 83, C₁₁H₂₈N_{_B}r₂ requires C, 37 6, H, 8 04 %

Heptamethylene bis-trimethylammonium dibromide BTM7

Crystallized from ethanol and ether mp 113° Found C, 38 3, H, 8 44, H₂0, 7 86 C₁₃H₃₂N Br, 1 8H₂O requires C, 38 3, H, 8 70, H₂O 7 86%

Octamethylene bis-trimethylammonium dibromide
BTM8

Crystallized from ethanol Loses water above 120° but does not melt below 280°



SUMMARY

- (1) The following series of polymethylene bisquaternary ammonium dibromides have been prepared and tested for curare-like activity on the phrenic nerve-diaphragm preparation of the rat (n=number of carbon atoms in the polymethylenechain) —bis-trimethylammonium series, n=2, 3, 4. 5, 7, 8, 9, 10, 11, 12, and 13, bis-triethylammonium series, n=2, 3, 4, 5, 7, 8, 9, 10, and 13, bis-strychninium series, n=2, 3, and 5, bis-quinolinium series, n=3,5, and 10, bis-(phenyldimethylammonium) series, n=3 and 5
- (2) In the bis-trimethyl series, the salt with n=2is about twice as active as tetramethylammonium iodide, salts with n=3, 4, or 5 are only feebly active, activity increases from n=7 to n=9, salts with n=9, 10, 11, and 12 are all about 5-6 times as active as tetramethylammonium
 - (3) In the bis-triethyl series, salts with n=2 or 3 are relatively inactive, activity increases from n=4to n=13, the last member being somewhat more active than the bis-trimethyl member in which $n \approx 9$
 - (4) None of the members of the other three series was so active as the most active members of the bis-trimethyl series
 - (5) In the rabbit head-drop test the bis-trimethyl member with n=9 was nearly as active as tubocurarine chloride, the member with n=10 was about three times as active The bis-triethyl member with n=13 was about two-fifths as active as tubocurarine chloride
 - (6) Some bis-onium salts, particularly bis-triethylammonium, bis-strychninium, and bis-quinolinium,

augment the response of the rat diaphragm to maximal stimuli and inhibit the cholinesterase of caudate nucleus (dog)

Our thanks are due to Dr. N K Dutta, who carried out the rabbit head-drop tests

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THE ANTIBACTERIAL ACTIVITY OF SOME SYNTHETIC COMPOUNDS RELATED TO PENICILLIN

BY

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Concurrently with attempts to synthesize penicillin, the chemistry and synthesis of which have been described by du Vigneaud Carpenter, Holley Livermore, and Rachele (1946) the anti-bacterial activity of compounds, or derivatives of compounds, known or postulated as parts of the penicillin molecule, has also been investigated

The compounds examined here may be conveniently classified into groups based on penicillamines (I) (Copp and Wilkinson, 1947a Duffin and Wilkinson, 1947a, b, Wilkinson 1947a), thiazolidine-4-carboxylic acids (II) (Wilkinson 1947b), oxazolones (III) (Copp and Wilkinson 1947b c) derivatives of glycine (IV), and a miscellaneous group of intermediate and associated compounds (Copp. 1947) (included in Table II)

Penicill n (with β-lactam structure)

Penicillin (with incipient azlactone structure)

II Thiazolidine-4-carboxylic acids

RESULTS AND DISCUSSION

The antibacterial activity of the compounds, added aseptically to nutrient broth, was estimated by exposing falling concentrations of the compounds, in powers of two, to a constant inoculum of *Str pyogenes*, CN 10 The results (Tables I and II) show that all compounds possess poor antibacterial activity *in vitro* when compared with penicillin (1,660 units per mg) Those penicillamine esters (Table I) which possessed antibacterial activity were inactivated, partially or completely, by the presence of 10 per cent of blood or serum The activity was not reversed by penicillinase and the morphological changes seen with penicillin were not observed

The compounds marked T (Tables I and II) were examined for acute toxicity and for therapeutic activity in mice infected with Str pyogenes, CN 10 All active compounds showed signs of toxicity within the range of 1–20 mg per 20 g mouse (05–10 g per kg), and no compound was chemotherapeutic Loss of antibacterial activity of the esters in the presence of blood and serum suggested that the absence of chemotherapeutic action might possibly be due to hydrolysis The

¹ Committee on Medical Research, O S R D, Washington and the Medical Research Council London Nature, 1945, 156, 766, and Science 1945 102 627 The Editorial Board, Monograph on the Chemistry of Penicillin, Science, 1947, 105, 653

TABLE I

The antibacterial activity of a series of compounds expressed as units of penicillin per mg. The organism is Streptococcus pyogenes, CN 10 Penicillin contains 1 660 u/mg. Compounds marked T were examined for toxicity and chemotherapeutic action

Compounds			ity expressed a icillin per mg	s	
Compounds	18 hrs	48 hrs	+10% blood	+10% serum	
I PENICILLAMINES S-Ethyl-cysteine S-Ethyl-cysteine methyl ester Cystine n-butyl ester dihydrochloride dl-Penicillamine d-Penicillamine dl-Penicillamine methyl ester hydrochloride dl-Penicillamine methyl ester hydrochloride dl-Penicillamine methyl ester hydrochloride dl-Penicillamine n-propyl ester hydrochloride dl-Penicillamine iso-propyl ester hydrochloride dl-Penicillamine iso-propyl ester hydrochloride T dl-Penicillamine iso-butyl ester hydrochloride T dl-Penicillamine iso-butyl ester hydrochloride T dl-Penicillamine iso-amyl ester hydrochloride T dl-Penicillamine n-hexyl ester hydrochloride T dl-Penicillamine n-hexyl ester hydrochloride T dl-Penicillamine n-hexyl ester hydrochloride T dl-Penicillamine n-hexyl ester hydrochloride T N-Phenaceturyl-dl-penicillamine S-Benzyl-dl-penicillamine ethyl ester T N-Phenylacetyl-S-benzyl-dl-penicillamine methyl ester T N-Formyl-S-benzyl-dl-penicillamine methyl ester T N-Acetyl-penicillamine methyl ester T N-Acetyl-penicillamine methyl ester T N-Acetyl-penicillamine methyl ester	1/128 <1/128 2/1/128 2/1/512 <1/512 <1/512 <1/512 <1/8 1/8 1/8 1/4 1/4 1/4 1/8 1 >8 >8 >8 >8 >8 >8 <1/1/28 <1/1/28 <1/1/28 <1/1/28 <1/512 <1/512 <1/512	<pre><1/128 <1/128 <1/128 2 <1/8 <1/256 <1/256 <1/8 <1/8 1/8 1/4 1/4 1/4 1/6 16 16 16 16 2 <1/1/128 <1/1/128 <1/1/128 <1/1/128 <1/1/128 <1/1/128 <1/1/128<!--1/1256</pre--></pre>	1/8 1/8 - 1/4 1/4 1/4 1/4 1/4 1/4	1/32 1/16 1/8 1/8 1/4 1/4 1/4 <1/256	

TABLE II

The antibacterial activity of further groups of compounds against Streptococcus pyogenes CN 10 expressed as units of penicillin per mg. Compounds marked T were examined for toxicity and chemotherapeutic action

Compounds	In vitro activity expressed as units of peni- cillin per mg		Compounds	In vitro ac expressed units of p cillin per	
	18 hrs	48 hrs		18 hrs	48 hrs
II THIAZOLIDINES 4-Carbomethoxy-2-phenyl-5 5- dimethyl-dl-thiazolidine hydro- chloride T 4-Carbomethoxy-2-aminomethyl 5 5-dimethyl-dl-thiazolidine hydrochloride 4-Carboxy-N-phenylacetyl-2 2 5 5- tetramethyl-dl-thiazolidine (ammonium salt)	1/32 <1/8	1/64 <1/8 <1/8	4-Carboxy-N-benzoyl-2 2 5 5- tetramethyl-dl-thiazolidine (ammonium salt) 4-Carbomethoxy-2-spiro-cyclohexyl- 5 5-dimethyl dl-thiazolidine hydro- chloride T 4-Carbo-n-amoxy-2-spiro-cyclohexyl- 5 5 dimethyl dl-thiazolidine hydro- chloride T	<1/8 1/64 1/4	<1/8 <1/32 <1/128

TABLE II—cont nued

	17	IBLE II-	-toni nusu		_t _
Compounds	In vitro a express units of cillin po	ed as peni-	Compounds	expres	activity ssed as of peni- per mg
)	18 hrs	48 hrs		18 hrs	48 hrs
II THIAZOLIDINES—continued		1	Triglycylglycine n-butyl ester T	<1/32	<1/32
4-Carbomethoxy-2-carbethoxymethyl-	i 1	1	Phenylglycine T N-Formyl-glycine methyl ester T	<1/512 <1/512	<1/512 $<1/512$
2 5 5-trimethyl-dl-thiazolidine			N-Formyl-glycinamide T	21/512	₹1/512
hydrochloride T 4-Carbomethoxy-N-benzoyl-	1/32	<1/128	a-Formyl-N-benzoyl-glycine	!	. 12.56
2 2 5 5-tetramethyl-dl-thiazoli-		,	ethylthioester T N-Benzoyl-a-ethylthiomethylene-	1/128	1/256
dine T	1/128	<1/128	glycinebenzylthio ester T	<1/512	<1/512
4-Carboxy-N-formyl-2 2 5 5- tetramethyl-dl-thiazolidine	<1/8	<1/8	N-Methyl-glycine ethyl ester hydro-	}	1.250
4-Amido-N-formyl-2 2 5 5-		•	chloride (Sarcosine ethyl ester) T N-Benzylglycine ethyl ester	<1/512	<1:256
tetramethyl-dl-thiazolidine T	<1/256	<1/256	hydrochloride T	<1/512	<1/256
n-Butoxy-N-formyl-2 2 5 5- tetramethyl-dl-thiazolidine T	>1	1/2	N'-(a-Formyl-N-benzoyiglycyl)-	1,1256	1/128
4-Carbomethoxy-N-formyl-	,		 p-aminobenzenesulphonamide T N'-(N-Benzoyl-a-ethylthio-methylene- 	1/256	1.120
2 2 5 5-tetramethyl-dl-thiazoli- dine	<1/128	<1/128	glycyl)-p-aminobenzene-		
4-Carbethoxy-N-formyl-2 2 5 5-		1	sulphonamide	1	1/8
tetramethyl-dl-thiazolidine T	<1/128	<1/128	glycyl)p-aminobenzenesulphon-		!
III Oxazolones	•		amide T	1/16	1/8
Δ"(4'-carboxy-5 5'-dimethyl-			MISCEI LANEOUS		, 1
thiazolidine)-2-phenyl-4-methyl-5- ovazolone	<1/128	<1/128	n-Butyl phenaceturate	1/128	<1/128
\(\frac{1}{4'-\text{carbomethoxy-5}} \) 5'-dimethyl-		1	n-Amyl phenaceturate	1/1/20	1/128
thiazolidine)-2-benzyl-5-oxazolone T	<1/128	<1/128	Ethyl α -amino- $\beta\beta$ -dimethylacrylate T	1/32	1/16
2-Phenyl-4-carbethoxymethyl- aminomethylene-5-oxazolone T	<1/512	<1/256	Methyl α-benzamido-ββ-dimethyl- acrylate T	<1/32	<1/16
2-Phenyl-4-benzylthiomethylene-		1	' Ethyl α-(N-benzylbenzamido)-β-	(
5-oxazolone T 2-Phenyl-4-(4 -aminobenzene-	<1/512	<1/256	hydroxyacrylate T Ethyl α-(N-methylacetamido)-β-	<1/512	<1/256
sulphonamido)-methylene-5-oxazolone	<1/128	<1/128	benzylaminoacrylate T	1/512	1/512
2-Phenyl-4-(2 -carboxyanılıno)- methylene-5-oxazolone	1 < 1/128	<1/128	Ethyl α-(N-methylbenzamido)-β- hydroxyacrylate T	-1/512	-1/513
2-Phenyl-4-(4 - aminoanilino)-	1		Mandelylalanine T	<1/512 <1/512	<1/512 <1/512
methylene-5-oxazolone	<1/128	<1/128	Acetylmandelylalanıne	<1/512	<1/512
2-Phenyl-4-(4 -amino-4 -diphenyl- aminomethylene)-5-oxazolone	<1/128	<1/128	Phenylaminoacetylalanine T $N-\Delta^a$ -Hexenoylalanine (ammonium	<1/512	<1/512
2-Phenyl-4-(4 -carbethoxy-	ł	1	(salt) T	<1/512	<1/512
anılınomethylene)-5-oxazolone 2-Phenyl-4-(3 -amınoanilıno-	<1/128	<1/128	Hippurylamide T Ethyl-N-benzyl hippurate T	<1/512 1/512	<1/512 <1/512
methylene)-5-oxazolone	<1/128	<1/128	N-Benzylhippuric hydrazide T	1/512	<1/512
2-Phenyl-4-(ethoxymethylene)-5- oxazolone	<1/8	<1/8	5-Carbomethoxy-2-phenyl- tetrahydro-1 4-thiazone-3		
2-Phenyl-4-(athylthiomethylene)-5-			5-Carboxy-2-phenyltetrahydro-	1/32	1/16
oxazolone T 2-Phenyl-4-(benzylthiomethylene)-5-	<1/64	<1/128	1 4-thiazone-3 (ammonium salt) T	<1/32	<1/16
oxazolone T	<1/512	<1/512	bis-Phenylchloracetyl-cystine dimethyl ester T	<1/32	<1/16
IV GLYCINE ESTERS	-		Benzyl mercaptan T	<1/32	<1/16
Glycine methyl ester hydrochlorideT	<1/32	<1/32	Tribenzylthiocarbinol T 2-Benzoylamino-3-pyrazolone T	<1/512	<1/512
Glycine ethyl ester hydrochloride T	<1/32	<1/32	Methyl norpenicillenate T	$ \frac{1}{128} $ $ < 1/512 $	<1/128 <1/512
Glycine n-propyl ester hydrochlorideT Glycine n-butyl ester hydrochloride T	<1/32 <1/32	<1/32 <1/32	Sodium norpenicillenate T 3-Keto-4-carbethoxy-4-phenyl-	<1/512	<1/512
Glycine iso-butyl ester hydrochloride			Δ^{α} -pentenoic acid T	1/128	1/32
Glycine <i>n</i> -amyl ester hydrochloride	<1/32	<1/32	Formamide T	<1/128	<1/128
T	<1/32	<1/32	dl-Valine n-butylester hydrochloride T	1/128	
	· ·		1	1/120	1/256

observation that mice given toxic doses of dl-penicillamine-n-butyl ester hydrochloride by the intraperitoneal route were first anaesthetized and only later succumbed to the convulsions characteristic of penicillamine added verisimilitude to this possibility. Comparisons of the acute toxicity of dl-penicillamine-n-butyl ester hydrochloride and its constituents were made in groups of ten mice. The results (Table III) suggest that, when due allowance is made for the rate of hydrolysis, the acute toxicity of the compound approximates to that of the mixture of its constituent parts

TABLE III

The average lethal doses of *dl*-penicillamine-*n*-butyl ester hydrochloride and its constituents when administered intraperitoneally to groups of 10 mice

Compound '	LD50 ± SD (mg per 20 g)
dl-Penicillamine hydrochloride dl-Penicillamine hydrochloride + n-butyl	40±06
alcohol	10.5 ± 1.3 13.0 ± 1.6
dl-Penicillamine-n-butyl ester hydrochloride n-Butyl alcohol	350±16 350±57

SUMMARY

1 Some penicillamine, thiazolidine, oxazolone, glycine and associated compounds have been examined for chemotherapeutic activity

- 2 The esters of penicillamine possess antibacterial activity in vitro, but their mode of action is not related to that of penicillin. They are inactivated in the presence of blood or serum, and evidence is presented which indicates that this may be due to hydrolysis
- 3 The more active compounds, when administered by the intraperitoneal route, were acutely toxic in small doses to mice
- 4 None of the compounds possesses chemotherapeutic value

We have to thank Drs F C Copp, W M Duffin, S Smith, and S Wilkinson of the Wellcome Chemical Research Laboratories for the compounds examined, Prof A H Cook for the specimen of 2-benzyl Δ $\frac{2}{3}$ -(4-carbomethoxy 5' 5'-dimethylthiazolidine) 5-oxazolone, and Mr M W Cheeseman for technical assistance in the investigations

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STUDIES ON THE BLOOD-BRAIN BARRIER I THE BASIS OF DOSAGE FOR ANIMALS OF VARIOUS WEIGHTS

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According to common belief, the cerebral bloodvessels are more permeable in the young than in the adult animal If any difference in permeability were such that the young blood-vessels allowed the passage of substances normally held back completely in older animals (e.g., many acid dyes), comparatively simple experiments would demonstrate it If, however, the difference were merely one of degree, it would be apparent only if a satisfactory basis were reached for adjusting, to animals of very dissimilar body-weight, the dosage of a dyestuff or drug intended to pass these blood-vessels and reveal its presence in the nervous tissues by staining, by pharmacological effects, or by other means None of our colleagues could state by what computation the adjustment should be made, and Clark (1937), discussing the dosage of drugs in general, wrote "there is no satisfactory method of relating dosage to body-weight when the latter varies extensively" The purpose of the present work was to inquiré whether any general rule of dosing could be established for causing effects of equal intensity in the nervous tissues of animals of wholly dissimilar weight (and hence age)

Should dosage be related to the weight of the brain? As Table I shows, the weight of the brain

relative to the body-weight is enormously greater in the young than in the adult mouse. To a somewhat lesser extent, the same is true of the rabbit. Although, other things being equal, the concentration of drug in an organ is likely to depend on the concentration in the blood and not on the size of the organ, a consideration of the possible variables involved suggested that these were so numerous that it was advisable to settle the matter by direct experiment

We had carried out preliminary experiments in which the dosage of dyestuffs and drugs was related to the body-weights or brain-weights of mice of different ages, or was calculated according to the metabolic-rate formula (metabolic rate ∞ body-weight ^{2/3}) The results showed that computation of the dose according to brain-weight invariably led to much more severe effects in Between the other computations, young animals the results did not permit of final choice, of 7 dyes and 5 convulsant drugs, some appeared to affect mice of different ages more uniformly when administered on the basis of body-weight, others when given according to the metabolic-rate formula

In the experiments described in the present paper, we adopted statistical methods of assess-

 $\label{table I} \textbf{TABLE} \ \ \textbf{I}$ body-weights and brain-weights in mice at different ages

	Number examined	Average body- weight	Limits	Average brain- weight*	Limits	Brain-weight as % of body-weight
Old mice	90	41 0 g	35 5-45 0 g	0 44 g	0 41–0 47 g	1 1
Young adult mice	174	20 0 g	19 0-21 0 g	0 42 g	0 40–0 42 g	2 1
Three-week-old mice	174	11 8 g	10 0-14 0 g	0 40 g	0 39–0 42 g	3 4
Two-week-old mice	84	5 6 g	not recorded	0 36 g	not recorded	6 4

ment Because of the very variable response afforded by different animals, and of subjective errors in assessing results, dves seemed unsuitable for the purpose We used, therefore, three convulsant drugs (cocaine, strychnine, and picrotoxin) and compared the dosage-mortality curves for mice of three different age-groups, we chose these particular convulsants partly because they are believed to act at quite different levels of the central nervous system, and partly because they have been used previously in experimental work on epilensy and the permeability of the blood-brain barrier (Cobb. Cohen, and Nev. 1938, Aird, 1939, Aird and Strait, 1944) In other tests we estimated chemically the amount of sulphanilamide passing into the brains of similar animals

METHODS

Mice were selected according to weight. The weight of a mouse is a fairly precise indication of its age only if conditions of maintenance have been standardized and, at any rate in younger mice, the sizes of the litters

have been reasonably constant. For the present purpose we used only mice from litters of two or three animals. These were raised from the age of two weeks under conditions as nearly standardized as possible with the prevailing standards of labour carefully supervised. Under these circumstances, the relation between weight and age was approximately as follows.

12 g $-2\frac{1}{2}$ weeks 20 g -5-6 weeks, 35 g -4 months

Before experiment, all mice were kept at a constant temperature ($\pm 5^{\circ}$ F) for at least three days. On the day of experiment we checked each cage for the pre sence of surplus food and water. The drugs were injected intravenously, for mice of 20 g (± 0.5 g.)* dissolved in 01 cc distilled water (02 cc with sulphanilamide) and introduced in exactly 10 sec (20 sec with sulphanilamide) timed with a stop-clock For mice of the other weights used (12 g ± 0.5 g and 35 g ± 1.0 g) the volume of the inoculum was adjusted to the body-weight, the time occupied in injection remained the same

TABLE II

MORTALITIES IN GROUPS OF 10 MICE INJECTED INTRAVENOUSLY WITH VARIOUS CONVULSANT DRUGS

For mice of 20 g the drugs were dissolved in 01 c c distilled water and injected in exactly 10 sec For mice of 12 g and 35 g the volume was adjusted to the body-weight, the time of injection remained the same

		12 g mice					20 g mice					35 g mice			
Drug	Dose	Dose Mortalities Expt No			Dose	Mortalities Dose Expt. No				Dose					
	μg	1	2	3	4	μg	1	2	3	4	μg	1	2	3	4
Strvchnine hydrochloride	4 5 6 7 8 10	0 0 3 3 7	1 5 6 8 9	1 3 5 7 9	1 3 5 7 9	7 8 9 10 11 12 14 16	0 0 1 2 5	0 0 3 5 7	1 5 7 8 10	1 3 6 8 10	12 15 18 21 24	0 1 1 4 10	0 0 2 5 9	0 2 2 5 9	0 1 2 5 9
Cocaine hy drochloride	150 200 250 300 350 400 450 500	0 0 5 , 9	0 2 5 6 8 10	0 2 4 6 8 10		200 300 400 500 600 700	0 0 2 5 9	0 2 6 9 10	1 2 5 9 10		300 400 500 600 700 900	2 2 4 7 7	1 3 -5 6 8 10	1 3 5 6 8 10	
Pierotoxin	25 30 40 50 60 70	2 4 4 7 9	0 3 5 8 10 10	0 2 5 7 9		50 60 70 80 90 100	3 6 9 9	0 2 6 8 9	0 3 5 8 9 10	_	50 70 90 100 110 130 160	0 4 8 10 10	0 1 2 6 10	0 1 2 6 9	

^{*}In this laboratory mice of 20 g are used in most experiments and are taken as the standard when comparison between various age-groups is made.

TABLE III

Probit-log dose relationships for mortalities in mice from convulsant drugs LD50 values, average slopes, and their standard errors

	Weight of mice				
Drug	12 g	20 g	35 g		
Strychnine slope	8 41 ± 1 23	9 56 ± 1 47	11 80 ± 1 68		
LD50 in mg	0 0069 ± 0 0002	0 01 L3 ± 0 0003	0 0203 ± 0 0005		
Cocaine slope	9 62 ± 1 46	10 22 ± 1 58	5 38 ± 0 85		
LD50 in mg	0 314 ± 0 010	0 472 ± 0 016	0 508 ± 0 024		
Picrotoxin slope	6 27 ± 0 90	12 00 ± 1 71	9 00 ± 1 30		
LD50 in mg	0 0419 ± 0 0016	0 0680 ± 0 0017	0 0891 ± 0 0039		

In the experiments with convulsant drugs groups each of 10 mice received doses selected to cover as nearly as possible the whole range of mortalities from 0 to 100 per cent, with at least three values between the limits. We repeated this procedure three or four times on different days, with any variations of dosage suggested by previous experiments.

In the experiments with sulphanilamide, groups each of 3 mice received the drug and were killed 20 min later. The choice of the time of autopsy was determined by experiments to be reported later. We estimated the amount of sulphanilamide in plasma and brain by the method of Rose and Bevan (1944). The experiment was repeated at approximately weekly intervals.

RESULTS

(a) Convulsant drugs

Table II presents the experimental data for the convulsant drugs used

The usual method of graphical representation of such data is to express the percentage mortalities as probits and plot these against the logarithm of the dose (Finney, 1947) A statistical analysis of the data of Table II shows that on this scale the dosage-mortality curves for all drugs are linear The slope of a particular line is thus a measure of the individual variation of the mice to the drug in question (Finney) Further analysis of the data shows that, for each age-group and each drug, the slope of the line does not differ significantly in repeat experiments, indicating that individual variation remained the same throughout the period of experiment—ie, for at least several days and in more than one batch of mice Finally, the LD50 values did not differ significantly from one experiment to another This is not a necessary condition of a satisfactory experiment, but it

TABLE IV

LD50 values and their standard errors expressed in terms of dose per 20 g mice (mg/mouse) on body-weight, metabolic-rate, and brain-weight formulae

Basis of assessment of dose	Drug	Weight of mice				
	}	12 g	20 g	35 g		
Bodv-weight mg./20 g	Strychnine Cocaine Picrotoxin	$\begin{array}{c} 0.0115 \pm 0.0003 \\ 0.523 \pm 0.017 \\ 0.0698 \pm 0.0027 \end{array}$	0 0113 ± 0 0003 0 472 ± 0 016 0 0680 ± 0 0017	0 0116 ± 0 0003 0 290 ± 0 014 0 0509 ± 0 0022		
Metabolic-rate formula	Strychnine Cocaine Picrotoxin	$\begin{array}{c} 0\ 0097\ \pm\ 0\ 0003\\ 0\ 442\ \pm\ 0\ 014\\ 0\ 0590\ \pm\ 0\ 0022 \end{array}$	$\begin{array}{c} 0.0113 \pm 0.0003 \\ 0.472 \pm 0.016 \\ 0.0680 \pm 0.0017 \end{array}$	0 0139 ± 0 0003 0 348 ± 0 016 0 0610 ± 0 0027		
Brain-weight	Strychnine Cocaine Picrotoxin	0 0072 ± 0 0002 0 330 ± 0 011 0 0440 ± 0 0017	0 0113 ± 0 0003 0 472 ± 0 016 0 0680 ± 0 0017	0 0194 ± 0 0005 0 485 ± 0 023 0 0850 ± 0 0037		

This table is derived from Table III Doses administered according to the metabolic-rate formula were in the following ratio 20 g mice 1 00, 35 g mice 1 46, and 12 g mice 0 71. The approximate mean brain-weights derived from Table I were respectively 0 42 g, 0 44 g, and 0 40 g. These values and the body-weights were used in calculating the above data. Thus for 12 g mice the LD50 for cocaine is 0 314. Expressed as dose per 20 g, mice on bodyweight basis, this becomes 0 314 \times 20/12 = 0 523. Calculated on the metabolic-rate formula, the dose would be

enables all the information for each drug contained in the experiments to be combined and represented by the average LD50, the average slope, and their standard errors (Finney)

By doing so we obtained the figures shown in Table III

Interesting observations can be made from the figures given for the slopes. With strychnine the slopes do not differ significantly for the three agegroups, this implies that the three age-groups have the same degree of variability in their reactions to strychnine With cocaine the old mice have a significantly lower slope than the other two agegroups, indicating that so far as their reaction to this drug is concerned the old mice vary amongst themselves more than do mice of the other age-For picrotoxin the reverse is true, the young mice vary more amongst themselves than do the other two age-groups Thus, comparing young and old mice, one may be more variable than the other with respect to one drug and less variable with respect to another The 20 g mice show up best for uniformity because with none of the drugs tested is either of the other age-groups significantly less variable

In Table IV the LD50 is expressed in terms of the three methods of assessing dosage For strychnine the LD50 values based on bodyweight are almost identical for the three agegroups. For this drug the best basis for calculating the dose is clearly by body-weight. For the other two drugs, none of the bases is completely satisfactory. There is reasonable agreement with picrotoxin for metabolic rate, but the body-weight basis is better when comparing young and 20 g mice. With cocaine the only reasonable agreement is for brain-weights when comparing old and normal, and for metabolic rate when comparing normal and young mice. For the other group in both instances alternative computation would result in gross error.

All these conclusions are based on the LD50 Since the slopes are not always the same for different age-groups, the above comparative results only apply for the LD50 Different comparative results would be obtained for other levels of mortality, but the conclusion will hold substantially for levels between 20 and 80 per cent

(b) Sulphanilanude

Table V shows the concentrations of sulphanilamide in the brains of mice of different weights Obviously, to some extent, the amount of drug in the brain must depend on that in the plasma, and

TABLE V

AMOUNTS OF SULPHANILAMIDE PRESENT IN THE BRAINS OF YOUNG AND OLD MICE INJECTED INTRAVENOUSLY

The first line for each dose refers to one experiment, the second to another, and the third to the last experiment

Doses for 12 g mice (1) 0 6 mg (2) 1 3 mg (3) 2 0 mg ,, ,, 20 g ,, (1) 1 0 mg (2) 2 0 mg (3) 3 0 mg ,, ,, 35 g ,, (1) 2 0 mg (2) 3 5 mg (3) 5 0 mg

For 20 g mice the above doses were contained in a volume of 0.2 c c injected in exactly 20 seconds of other weights the volume was adjusted to the body-weight, the time of injection remained the same Ratio = concentration in brain/concentration in plasma

Amounts are given in mg per 100 ml plasma or 100 g brain, each pair of entries represent the concentration of sulphanilamide in the plasma and brain of a single mouse

	Weight of mice								
Dose		12 g			20 g			35 g.	
	Plasma	Brain	Ratio	Plasma	Brain	Ratio	Plasma	Brain	Ratio
1	3 54 3 75 3 77 Mean 3 69	3 41 3 10 3 75 3 42	0 93	3 98 4 17 3 15 3 77	3 03 2 97 2 47 2 82	0 75	4 56 4 75 4 54 4 62	2 92 3 47 3 35 3 25	0 70
2	6.27 5 53 8 14 Mean 6 65	4 91 4 92 5 10 4 98	0 74	5 61 8 38 7 37 7 12	4 83 5 91 5 43 5 39	0 76	8 47 8 20 6 84 7 84	5 62 5 74 6 16 5 84	0 74
3	11 88 15 32 12 91 Mean 13 37	9 86 10 88 9 45 10 06	0 75	14 07 13 98 10 92 12 99	11 67 8 06 7 77 9 17	0 71	12 43 10 84 11 44 11 57	8 14 7 66 7 69 7 83	0 68

the ratio between these two quantities would appear to be the more signifi-These ratios are fairly cant figure constant except for the lowest dose in young mice The last may well represent a testing error resulting in an abnormally high value for the concentration in the brain, it is not however, sufficiently different from the other figures to warrant rejection of the re-The geometric mean of all nine ratios is 0.75, which may be taken as the ratio, 20 minutes after dosing, for a wide range of doses and weight of The reason for taking the geometric and not the arithmetic mean will be apparent in the following analysis

Analysis of results

The mean concentrations in Table V have been plotted on log scales in Fig 1-ie, log concentration against log dose Some curvature is apparent in each graph, but it is clear that all curves are substantially parallel and that the relation between the plasma curves for young, normal, and old mice is similar to that for the brain curves If the curves are in fact parallel and occupy relatively the same position within both sets (subject of course to experimental error), then the interpretation is greatly simplified If the curves were not parallel, no uniform basis of calculating the dose would be possible, because the basis would then depend on the magnitude of the dose If, for instance, the curves converged for low doses (and diverged for high), the ratios of the doses for young, normal, and 'old mice giving the same concentration of drug in the brain would be closer together for low than for high doses The assumption that the curves are parallel was made, therefore, as a working hypothesis, to be subject to a rigorous statistical test at a later stage assumption could be justified, a uniform basis for assessing doses would exist, this basis would be the ratio of the doses which yield the same concentration of drug in the brain (or plasma) for all three age-groups

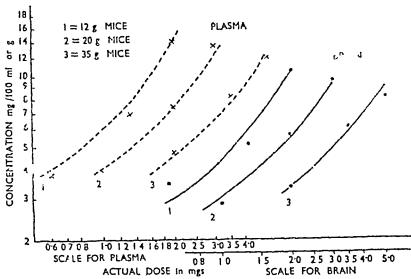


FIG 1—Relation between dose of sulphanilamide and concentration in plasma and brain for young, normal, and old mice

The assumption is embodied by representing the six curves by the equations in Table VI

Fitting these lines to the log of the values of Table V by the method of Least Squares, we obtain the following values for the constants $m_1=0.169$, $m_2=0.421$, a=0.726, A=0.602, b=0.909, c=1.233 These refer to the origin -0.222 for plasma and brain, and the theoretical curves are plotted in Fig. 1, it will be seen that the fit is reasonably good. The next step is to assess whether this fit is consistent with the experimental error. To do this we calculate for each animal the quantities.

 $Y = \log$ concentration in plasma + \log concentration in brain

 $Z=\log$ concentration in plasma $-\log$ concentration in brain

The variation within the groups of three mice for each dose and age group represents the experimental error for these quantities. An analysis of variance of Z shows that no significant variation arises between groups. It follows that the ratio of concentration in the brain to the concentration in the plasma may be assumed constant for all doses and age-groups of mice examined

The fitted lines for the quantities Y for the three age-groups are derived by summing the corre-

TABLE VI
Sulphanilamide in the brains of mice formulation of equations for log concentration of sulphanilamide v log dose

	Plasma	Bram
Young Normal Old	$y = a + bx + cx^{2}$ $y = a + b(x - m_{1}) + c(x - m_{1})^{2}$ $y = a + b(x - m_{2}) + c(x - m_{2})^{2}$	$y = A + bx + cx^{2}$ $y = A + b(x - m_{1}) + c(x - m_{1})^{2}$ $y = A + b(x - m_{2}) + c(x - m_{2})^{2}$

sponding equations for the plasma and brain. The variance of the nine group means about these lines is 0 0065 based on 4 degrees of freedom. This has to be compared with the experimental error variance. We calculate that the variance of Y within groups of three mice is 0 0093 based on 18 degrees of freedom, accordingly the error variance of the group means is 0.0093/3=0.0031. The variance about the fitted lines is not significantly greater than this. The fit of the lines is, therefore, adequate and the working assumptions justified

We have now shown that within the experimental error, the ratio of the concentration in the brain and plasma is the same for all doses and weight of animal within the range examined, and also that the curves connecting the concentration in the plasma (and brain) with log dose for the three age-groups are parallel

The effect of age (or weight) of mouse on the concentration in the plasma and brain is therefore given entirely by the two constants m_1 and m_2 calculated previously—i.e., $m_1=0.169$, $m_2=0.421$. These are in terms of log dose, their antilogs are 1.476 and 2.637. The relation between the doses to give the same concentration for young, normal, and old mice is, therefore, 1.00 to 1.476 to 2.637 respectively. Expressed as normal=100, we get 67.100.178

In Table VII we compare these ratios with the three bases for assessing dose

The ratios found agree almost entirely with the body-weight basis of assessment. The experimental error in the ratios found is unlikely (p=0.05) to exceed \pm 12%. We can therefore confidently exclude the other two bases of assessment of the dose, since the experiment is on a sufficient scale to discriminate conclusively between the three bases and thus gives a clear-cut result. Fewer animals would have been insufficient and a larger number unnecessary. The conclusion is that the correct basis of assessment of dose is by body-weight.

TABLE VII
Sulphanilamide in the brains of mice ratio of dose per mouse calculated in various ways compared with doses causing equal concentrations of the drug in the brain

Basis of assessment of dose	Weight of mice			
Dasis of assessment of dose	12 g	20 g	35 g	
Found experimentally Body-weight Brain-weight Metabolic-rate formula	62 67 95 72	100 100 100 100	169 178 105 146	

An estimate of the constant brain/plasma concentration may be obtained from a and A calculated above. The difference A-a=1 876 which corresponds to an antilog of 0.75. This, of course, is identical with the geometric average determined previously from Table III, and is the justification for using the geometric in place of the arithmetic mean of the ratios.

SUMMARY AND CONCLUSIONS

The foregoing experiments sought to determine whether there exists any uniform basis of dosage, for mice of different weights, of drugs penetrating the blood-brain barrier. As an indication of their presence in the nervous tissue, we determined the mortality curves of three convulsant drugs, strych nine, cocaine, and picrotoxin. We also estimated chemically sulphanilamide passing into the brain All the drugs were given intravenously under rigidly standardized conditions, thereby eliminating factors concerned with absorption from the all mentary canal or from the tissues

Although, proportionately to the body-weight, the brain is relatively very much heavier in the young animal, dosage on the basis of brain-weight nearly always results in the administration of too Apart from this state large amounts of drug ment, no general rule can be formulated strychnine and sulphanilamide, dosage on the basis of body-weight gave results most nearly uniform With picrotoxin, dosage in the various mice according to the metabolic-rate formula gave reasonably uniform results over the complete range of weights, but dosage on the basis of body weight gave closer correlation between immature mice and young adults With cocaine no basis of dosage appeared satisfactory for all weights Com putation according to the metabolic-rate formula yielded greatest uniformity between immature mice and young adults, according to the brain-weight between young adults and old mice

In the absence of any uniform basis of dosage for mice of widely different weights, the recognition of small differences in the permeability of the cerebral blood-vessels of young and old animals would be a matter of considerable difficulty,

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THE DECARBOXYLATION OF β-3 4-DIHYDROXYPHENYLSERINE (NORADRENALINE CARBOXYLIC ACID)

BY

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 β -3 4-Dihydroxyphenylserine is an amino-acid structurally closely related to noradrenaline. In this paper it will therefore be called "noradrenaline carboxylic acid"

The amino-acid was first prepared by Rosenmund and Dornsaft in 1919, and again by Guggenheim (1940), who found that in rabbits it was without action on the arterial blood pressure after subcutaneous injection of 0.1 g/kg. The acid has recently been prepared by Dalgleish and Mann (1947), together with the corresponding N-methyl-amino-acid, "adrenaline carboxylic acid"

Rosenmund and Dornsaft (1919) suggested that the amino-acid might be a precursor of adrenaline in mammals, but they did not support this suggestion by experiments

A scheme of adrenaline synthesis has been discussed which assumes the decarboxylation of L-3 4-dihydroxyphenylalanine (Blaschko, 1939, 1942, 1948), a reaction catalysed by the enzyme dopa decarboxylase (Holtz, Heise, and Ludtke, 1938) This enzyme does not act on N-methyl-3 4-dihydroxyphenylalanine (Blaschko, 1939), but it is not known how the introduction of a hydroxyl group in the β -position affects substrate specificity We have therefore examined noradrenaline carboxylic acid as a possible substrate of mammalian decarboxylases

The mammalian dopa decarboxylase is related to the bacterial L-tyrosine decarboxylase, and we have therefore included experiments on the decarboxylation of the amino-acid by the bacterial enzyme, these experiments have enabled us to establish the stereochemical configuration of 3 4-dihydroxyphenylserine

MATERIALS AND METHODS

For the noradrenaline carboxylic acid we are grateful to Dr F G Mann, FRS Two samples of noradrenaline hydrochloride were used for the bio-

logical assay the first was a racemic preparation, known as Arterenol, which was obtained from the IG Farben-Industrie more than ten years ago for the second sample, a specimen of the laevorotatory stereoisomer, we are grateful to Dr M L Tainter

The tissue extracts—from guinea-pig's kidney and suprarenal gland—were prepared as previously described (Blaschko, 1942) All our manometric experiments were conducted in an atmosphere of nitrogen

An acetone-dried preparation was used for the study of the bacterial L-tyrosine decarboxylase. We are grateful to Prof I C Gunsalus for a strain of Streptococcus faecalis R (ATCC 4083), the bacteria were grown on a medium similar to that described by Bellamy and Gunsalus (1945), but with an addition of 0.5 mg/litre of pyridoxal (Merck)

The pharmacological assay was carried out on the arterial blood pressure of the spinal cat, the rat's uterus preparation used in one experiment has been described by Garcia de Jalon, Bayo Bayo and Garcia de Jalon (1945)

EXPERIMENTS

(1) Experiments with guinea-pig's kidney extract

The guinea-pig's kidney gives extracts of high dopa decarboxylase activity, we compared the rate of decarboxylation of the racemic 3 4-dihydroxy-phenylserine with that of DL-3 4-dihydroxy-phenylalanine The manometric set-up was as follows

Maın Flask	Fla-k 1 1 6 ml guinea-	Flask 2 pig kidney extrac	Flask 3 at (in all flasks)	
Side Bulb	0 4 ml water	0 4 ml M/100 DL-3 4-dihy- droxyphenyl- alanine	0 4 ml M/100 noradrenaline carboxylic acid	
Incubation temperature 37 5° C				

From one molecule of DL-dihydroxyphenylalanine half a molecule of carbon dioxide should be formed, as it is known that the decarboxylase is stereospecific and will form carbon dioxide only

from the L-isomer The amount of carbon dioxide expected to be formed from 0.4 ml of M/100 DL-amino-acid is 44.8 μ l

From 3 4-dihydroxyphenylalanine the theoretical amount of carbon dioxide was formed within the first nine minutes, and the reaction then came to a standstill There was no formation of carbon dioxide from noradrenaline carboxylic acid

(2) Experiments with extracts from the guineapig's suprarenal gland

There was no formation of carbon dioxide when extracts from the suprarenal gland were incubated with either 3 4-dihydroxyphenylalanine or nor-adrenaline carboxylic acid

(3) Experiments with Streptococcus faecalis

It is known that the L-tyrosine decarboxylase of Strep faecalis also acts on 3 4-dihydroxyphenylalanine (Epps, 1944) It seemed therefore of interest to find out how the introduction of a hydroxyl group in the β -position of the side chain would affect substrate specificity

Two experiments were carried out in which the amino-acid was incubated with the bacterial pre-Carbon dioxide was formed in both Experiments This suggested that noradrenaline had been formed by the enzyme The pressor activity of the solutions was therefore estimated on the blood pressure of the spinal cat In the first experiment the activity was assayed against (+)-noradrenaline and in the second against the laevorotatory isomer Since the results obtained in the two experiments showed satisfactory agreement, only the second experiment will be described ın detail

(a) Incubation—A suspension was prepared containing 10 mg of the acetone-dried bacteria in 1 ml of distilled water

Two manometer flasks were set up as follows

1 440 111411	officiel flasks we	ie see up as follows
-	Brank	Test
Ma n Flask	10 ml bacterial +01 ml M/1 a +02 ml water	suspension cetate buffer, pH 5 5 in both flasks
Side Bulb	02 ml water	0 2 ml M/12 5 nor adrenaline carboxy- lic acid

A third flask was set up in which the noradrenaline carboxylic acid in the side bulb was replaced by 0.2 ml of a suspension of L-tyrosine. This flask was used in order to enable us to relate the rate of decarboxylation of noradrenaline carboxylic acid to that of tyrosine.

The incubation was carried out at a temperature of 28 5° C

Separately, a smaller amount of L-tyrosine was incubated with the bacterial preparation under the same conditions, this was done in order to determine how much of the carbon dioxide formed had been retained as bicarbonate. The retention was found to amount to about 6 per cent, this figure was therefore used as a correction in the main experiment.

Formation of carbon dioxide occurred when noradrenaline carboxylic acid was added to the enzyme. The rate of decarboxylation was steady for the first hour, but then slowed down, the reaction had almost come to a standstill when the incubation was stopped after 315 min. By that time 168 μ l of carbon dioxide had been formed. On the assumption that half a molecule of carbon dioxide is formed from one molecule of noradren aline carboxylic acid 179 μ l should be formed. Thus the observed formation of carbon dioxide represents 94 per cent of theory. The time course of the reaction is shown in Fig. 1

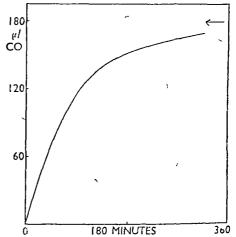


Fig 1—Decarboxylation of noradrenaline carboxylic acid by Streptococcus faecalis R Abscissa time in minutes, Ordinate μl CO₂ formed The arrow denotes half a molecule of CO₂ per molecule of amino-acid added

After the last reading the contents of the blank and test flasks were each pipetted into a centrifuge tube Each flask was washed out with two successive portions of 0.25 ml of water. For the pharmacological assay it was assumed that any active material formed was now contained in a total volume of 2.0 ml.

The suspensions were centrifuged for 15 min, in order to remove the bacterial debris, and the supernatant fluids were used for the assay

(b) Assay —The manometric experiment suggested that amine formation from noradrenaline

carboxylic acid had occurred It was desirable to establish the identity of the amine formed with noradrenaline and to determine its pharmacological activity. The assay was carried out on the spinal cat and the identity of the pressor principle was confirmed on the rat's uterus

Spinal cat 32 kg of The test was used in a dilution 1 in 25. The standard of laevorotatory noradrenaline was made up in a solution of the blank so that the final concentration of the blank was also 1 in 25.

The pressor activity of the test sample is shown in Fig 2, the tracing shows that 05 ml of the test

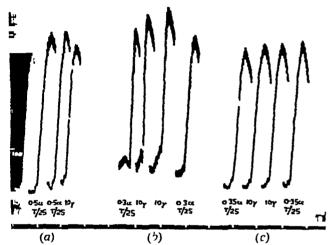


Fig 2.—Assay of pressor activity produced from nor-adrenaline carboxylic acid by the bacterial enzyme Arterial blood pressure of the spinal cat 10 µg of synthetic (—)-noradrenaline were (a) less active than 0.5 ml, (b) more active than 0.3 ml, (c) about as active as 0.35 ml of the test solution, diluted 1 in 25

diluted 1 in 25, had a stronger, and 0 3 ml of the same solution a weaker, pressor action than 10 μ g of (—)-noradrenaline The pressor action of 10 μ g of (—)-noradrenaline was found to be approximately equal to that of 0 35 ml of the test, diluted 1 in 25 The blank was without action on the blood pressure

(c) Calculation of result—The total amount of pressor activity formed in the experiment can be calculated from these data. The pressor action contained in 2 ml of the undiluted test was equivalent to more than 1 00 mg and less than 1 62 mg of (—)-noradrenaline, and about equal to 1 40 mg of (—)-noradrenaline

The total amount of amine expected to be present can be calculated from the amount of carbon dioxide formed, one molecule of amine is formed per molecule of carbon dioxide

R-CH(NH₂)COOH \longrightarrow R-CH₂NH₂+CO₂ The molecular weight of noradrenaline is 169 Therefore, 22,400 μ l of CO₂ correspond to 169 mg of noradrenaline, and 168 μ l of CO₂, the amount formed in the experiment, correspond to $\frac{169 \times 168}{22.400} = 1.27$ mg of noradrenaline

The agreement between the amounts found and expected is satisfactory and shows that the amine formed was in fact (-)-noradrenaline. The amine formed cannot have been (\pm) -noradrenaline

Similarly, it could be calculated that in the first experiment in which the activity was assayed against (±)-noradrenaline, the amount of amine formed was about twice as active as (±)-noradrenaline. In this experiment the assay on the cat's blood pressure was followed by an assay on the rat's uterus

(d) Rat's uterus assay — One horn of the uterus of a non-pregnant rat was suspended in a bath of capacity 5 ml at a temperature of 31° C. The composition of the Ringer's fluid was similar to that recommended by Garcia de Jalon et al. (1945) Submaximal contractions of the uterus were recorded, a dose of 5 μg of acetylcholine was added to the bath every 90 secs. The acetylcholine was present for 30 secs, and 60 secs were allowed for recovery. Adrenaline, noradrenaline, or the test solution was added to the bath 30 secs before the acetylcholine.

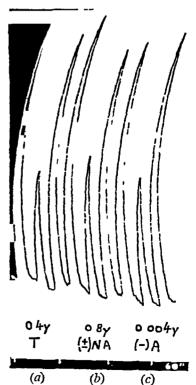


Fig. 3—Isolated uterus of the rat Each contraction was caused by 5 μ g of acetylcholine. The inhibitions were due to (a) the test solution in an amount calculated to contain 0.4 μ g of amine, (b) 0.8 μ g of (\pm)-noradrenaline, (c) 0.004 μ g of (\pm)-adrenaline

A dilution of the test was given calculated to contain $0.4~\mu g$ of (-)-noradrenaline Fig 3 shows that the inhibition observed was about equal to that after $0.8~\mu g$ of (\pm)-noradrenaline. The same result was obtained at three different dose levels. The Figure confirms West's (1947) finding that the rat's uterus preparation is many times more sensitive to adrenaline than to noradrenaline.

The result obtained on the rat's uterus preparation therefore confirms that of the blood pressure assay the amine formed is (-)-noradrenaline

DISCUSSION

In order to assess whether or not noradrenaline carboxylic acid can be considered as a likely precursor of adrenaline or sympathin, it is necessary to consider its structure. The acid differs from 3 4-dihydroxyphenylalanine in that it contains 2 asymmetric carbon atoms, one in position α and one in position β

HO H H H
$$C^{\beta} - C^{\alpha} - NH_{\mathbf{I}}$$
OH COOH

Noradrenaline carboxylic acid

Compounds of this kind have four different configurations and exist in pairs related as object and mirror image The four possible arrangements in space are shown in Fig 4, in which the α and β carbon atoms are replaced by regular tetrahedra The designations L and D are used for the α carbon atom the configuration L is that common to most naturally occurring aminoacids The two possible configurations of the β carbon atom are distinguished as l and d, this is the asymmetric carbon atom of adrenaline, the naturally occurring laevorotatory configuration is usually referred to as l-adrenaline

Dr Mann told us that the sample of noradrenaline carboxylic acid which we used in our experiments behaved like a simple racemic mixture and that it therefore contained only one pair of stereoisomers. The two pairs of stereoisomers are Dd-Ll and Dl-Ld

The experiments reported above enable us to determine the configuration of noradrenaline carboxylic

acid This is possible, because two stereospecific affinities are involved

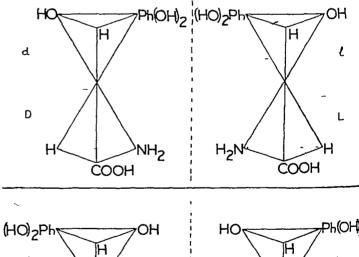
- (a) that of the bacterial amino-acid decarboxylases for the L-configuration of the α carbon atom, and
- (b) that of the plain muscle cell for the l-configuration of the β carbon atom

All known amino-acid decarboxylases, bacterial and mammalian, are stereospecific, and it can therefore be safely assumed that only the *L*-stereoisomer was decarboxylated in our experiments. This is borne out by the observation that approximately half a molecule of carbon dioxide was formed from one molecule of noradrenaline carboxylic acid.

The stereospecificity of the mammalian receptors has recently been studied by Tainter, Tullar, and Luduena (1948), who have shown that on the spinal cat's blood pressure the ratio of equiactive doses of laevorotatory to dextrorotatory noradrenaline is 1 25 to 1 33

Theoretically three structures are possible

(1) a racemic mixture of the configurations Dd and Ll, in this case one would expect (-) noradrenaline to be formed



D HONH2 H2N COOH

Fig 4—The four configurations of "noradrenaline carboxylic acid The alpha carbon atoms are represented by the lower, and the beta carbon atoms by the upper, tetrahedra

- (2) a racemic mixture of the configurations Dl and Ld, in this case one would expect (+)-noradrenaline to be formed
- (3) a mixture of all four configurations in this case one would expect (\pm) -noradrenaline to be (This possibility was already ruled out by Dr Mann's findings)

That the first of these is in fact the structure of the acid is proved by the pharmacological assay on the spinal cat. The L-amino acid was found to be almost quantitatively decarboxylated and to have given rise to (-)-noradrenaline

Rules for the nomenclature of amino-acids have recently been agreed upon by the editors of the Biochemical Journal, the Journal of the Chemical Society, and the corresponding American publications (1948) Rule 6 deals with amino-acids "with two asymmetric centres, but where internal compensation is impossible" Noradrenaline carboxylic acid is unlikely to be present in proteins, but it is so closely related to amino-acids found in proteins that it seems desirable to follow the rules laid down for these compounds The substance studied in this paper contains the configuration in which the L-amino-acid has the configuration related to naturally occurring laevorotatory adrenaline, and it is therefore proposed to use the designation 3 4-dihydroxyphenylserine for this specimen and to reserve the prefix allo- for the pair wh ch still awaits synthesis

3 4-Dihydroxyphenylserine has the configuration to be expected in a possible precursor of adrenaline, so that the negative results obtained with the mammalian decarboxylases cannot be ascribed to the use of the wrong stereoisomer but must be interpreted as meaning that this substance is unlikely to be a precursor of adrenaline or sympathin

SUMMARY

- (1) Noradrenaline carboxyl c acid is not a substrate of dopa decarboxylase
- (2) Extracts of guinea-pig's suprarenal glands do not contain dopa decarboxylase, and they do not decarboxylate noradrenaline carboxylic acid
- (3) Noradrenaline carboxylic acid was decarboxylated by an acctone-dried preparation of Streptococcus faecalis R, the amine formed was characterized as (-)-noradrenaline by pharmacological assay on the blood pressure of the spinal cat and on the rat's uterus

The authors are grateful to the Medical Research Council for a personal grant to one of us (G H S S).

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ACTIVATION OF PALLIDRINE

BY

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The purpose of this paper is to submit evidence that paludrine has little activity in preventing the development of malaria parasites in vitro, but that it develops such activity when administered to the mammalian or avian host or when incubated with minced liver tissue

Paludrine is N₁-p-chlorophenyl - N₅ - isopropyl b guanide

Tonkin (1946), in this laboratory, investigated the effect of adding paludrine to tissue cultures containing exo-erythrocytic forms of Plasmodium gallingceum and found that the parasites developed in the highest concentrations (2-5 mg per litre) tolerated by the macrophages Since this concentration is many times greater than that which is believed to be present in the plasma during human therapy (probably about 03 mg per litre), and since Davey (1946) had reported that paludrine was highly effective in curing the exo-erythrocytic infections of P gallinaceum in chickens, as well as the endo-erythrocytic ones, Tonkin's finding was surprising and led to further investigations, which were undertaken with both P gallinaceum and cynomolgi A preliminary note describing some of the results was published by Hawking (1947)

Experiments with P gallinaceum in tissue culture

The exo-erythrocytic forms of *P* gallinaceum were grown in tissue culture at 37° C by the technique described by Hawking (1945) and used by Tonkin (1946) to study the action of antimalarial compounds. The implants were obtained from the spleen of chickens, at the stage of infection when exo-erythrocytic parasites were present. In the first experiments the cultures were grown in the presence of normal serum for several days, until the development of parasites in them could

be demonstrated, the medium to be tested for antimalarial activity was then added and the fate of the parasites was observed by removing cultures at intervals during the next ten days for staining with Giemsa and examination in the usual way The protocol of a typical experiment is reproduced in Table I For this experiment serum was

TABLE I

The medium consisted of 20 per cent serum, 20 per cent embryo extract, and 60 per cent Tyrode's solution by volume. The paludrine serum in Nos 2, 3, and 4 was substituted for all or part of the normal serum. The paludrine in Nos 6 and 7 was added to the Tyrode's solution. On the 5th day the medium was changed, and fresh medium similar to that originally present was added. Each No represents two Carrel flasks and most of the observations are based on four cell-colonies (slips), two from each flask. The cultures had grown for 4 days before the experiment began, and the development of parasites in them had been demonstrated.

No	Contents	Presence of parasites in cultures					
190	Contents	2nd day	4th day	7th day			
1	Control— Normal serum 20%		-	Many			
2	Paludrine serum 20%	_	A few in one culture None in another	None—chick not infected			
3	Paludrine serum 7%	Some	None	None — chick not infected			
4	Paludrine serum 2%	Some	None	None			
5	Paludrine			Toxic to cells			
6	5 mg, per Paludrine			Many			
7	2 mg per l Paludrine 1 mg per l			Many			
			2				

obtained from a fowl to make up the normal fluid culture medium. The fowl was then given 300 mg paludrine per kg intramuscularly (a toxic dose), and blood was removed 2½ hours later to provide the "paludrine serum" (All quantities of paludrine in this work refer to paludrine acetate)

In this experiment paludrine added directly to tissue cultures had little or no action on the parasites unless the final concentration was so high (eg, 5 mg per litre in No 5) as to be toxic to the cells without which the parasites cannot grow, this reproduces the finding of Tonkin already men-On the other hand the addition of serum from a fowl treated with a large dose of paludring was highly active, and all the cultures were sterilized, mostly by the 4th day The survival of parasites for a few days, even in a medium which eventually kills them, is in agreement with the experience of Tonkin, who found that parasites persisted in the presence of sulphathiazole (5-50 mg per litre) for 4 days but that most were destroyed by the 5th day The death of the parasites in Nos 2 and 3 was confirmed by removing fluid from the flasks on the 7th day and injecting it into chicks, which did not become infected Probably the activity shown by the paludrineserum in this experiment was particularly high because of the large size of the dose of paludrine given to the fowl In other experiments the fowl was given 30 mg per kg 18 hours and 2 hours before the blood was taken, and the antimalarial action of the serum was not manifested when the concentration of serum in the flask was less than 5 per cent.

These results suggest that paludrine undergoes some modification in the fowl so that it develops an activity against the parasites in vitro which was not possessed by the original paludrine hypothesis was studied further by incubating paludrine with minced liver tissue and then testing its action on the parasites The liver was removed aseptically from normal rats and minced finely About 6 cc of minced liver was placed in each of two tubes containing 10 cc. of Ringer with 02 per cent (w/v) glucose, one tube contained 12 mg paludrine per litre, which when diluted 1 in 6 provided a final concentration of 2 mg per litre, and the other served as a control The tubes were incubated at 37° C. for 4 hours and then centrifuged, and the supernatants removed These provided the "liver extract" and "liver extract plus paludrine," the use of which is illustrated in the typical protocol reproduced in Table II In this experiment the fluids to be tested for antimalarial activity were added to the Carrel flasks when the cultures were first set

TABLE II

The medium consisted of 17 per cent serum, 4 per cent embryo extract and 79 per cent Tyrode's solution by volume The paludrine serum in Nos 3, 4, and 5 was substituted for all or part of the normal serum (A) The liver extract (with or without paludrine) in Nos 6, 7, 8, and 9 was substituted for part of the Tyrode's solution Each No represents two Carrel flasks and most of the observations are based on four cell-colonies (slips)

No	Contents	Concentra- tion % (v _i v)	Presence of parasites on 5th day
1	Serum A	17	Many
2	Control Serum B ₁ Control	17	Very many
3	Paludrine serum	17	None
4	B. Paludrine serum	4	Few and degener-
5	Paludrine serum B ₃	1	ate None
6	Liver extract	17	Very many
7	Liver extract + paludrine	17 + 2 mg per l	None in 2 cultures, 1 parasite in a 3rd culture, and 2 in a 4th
8	Liver extract + paludrine	4 +	Fair number
9	Liver extract + paludrine	0 5 mg per 1 1 + 0 12 mg per 1	Few

up, without allowing a preliminary period for growth (Tonkin, 1946) Serum A was obtained from a normal fowl and it was used as serum for all the cultures unless stated otherwise Serum B, was obtained from a fowl before it received paludrine, it was then given two intramuscular injections of 30 mg per kg 18 hours and 1 hour respectively before the "paludrine serum," B2, was with-In this experiment the paludrine serum showed fairly high activity, since the growth of parasites was prevented (completely or incompletely) by concentrations of 1-4 per cent Liver extract alone had no activity against the parasites, but paludrine which had been incubated with liver showed fair activity, preventing the growth of all but rare parasites when in a concentration originally of 2 mg per litre Actually, it is probable that much of the paludrine had been absorbed by the liver tissue and the real concentration of paludrine (and paludrine derivatives) was much less than this theoretical figure Other experiments (see Table I) have demonstrated that unchanged

paludrine is inactive in a concentration of 2 mg per litre

After the tissue culture experiments had been completed, chemical estimations were made on the concentration of paludrine present in the serum of two fowls, each of which had received two intramuscular injections of 30 mg paludrine per kg 18 hours and 1 hour respectively before the blood was withdrawn. The concentrations found were 11 mg and 27 mg per litre respectively, average 19±11 3 mg per litre As the serum constituted only 17 per cent of the fluid present in the flasks of Table II, the highest concentration of paludrine present in the medium containing "paludrineserum" (Flask 3) would be about 3 2 mg per litre. while the medium in Flask 5 (which contained only 1 per cent paludrine serum and which prevented growth of the parasites) would contain 0 19 mg per litre These figures may be compared with the concentration of 2 mg unchanged paludrine per litre which is inactive

Experiments with P cynomolgi

P cynomolei was cultured in vitro at 37° C by a modification of Bass and John's technique Briefly, about 15 cc blood was withdrawn from a rhesus monkey (A) infected with P cynomolgi at a time, preferably before noon, when most of the parasites (95 per cent) were present as small rings The blood was defibrinated by shaking with glass It was centrifuged, most of the serum was removed, the corpuscles being resuspended in the Sufficient 10 per cent (w/v) glucose remainder solution was added to the serum to make the concentration of added glucose 0.2 per cent cultures were made in small flat-bottomed tubes. 6 cm high by 0.6 cm internal diameter, which Each tube con were closed by rubber bungs tained 06 cc monkey serum, 01 cc Ringer's solution, and 005 cc suspension of parasitized red blood corpuscles The corpuscles formed a thin layer on the floor of the tube Serum A was obtained from Monkey A or from another normal

TABLE III

Serum A was obtained from an untreated monkey which provided the parasitized blood Serum B₁ was taken from a second monkey before it received paludrine Serum B₂ was taken from the second monkey at 11 a m, when it had received paludrine 50 mg 18 and 2 hours previously In tubes 9-11 the dilutions were made with serum A Tubes 12-19 contained serum A (0 6 c c) plus 0 1 c c of liver extract (incubated with or without paludrine 14 mg per litre) pure or in various dilutions made with Ringer At the beginning of the experiment all the parasites contained only one piece of chromatin, and 95 of them were young rings, there were no gametocytes

		Per cent distribution of parasites according to number of pieces of chromatin					
Tube	Contents	1	23	4-5	6–8	9–16	Degen erate
1 2	Control, serum A	26 32	22 18	12 14	16 10	24 26	0
3 4	Control, serum B ₁	28 36	22 16	18 4	10 16	22 28	0
5	Paludrine, added to serum A, 2 mg per l Paludrine, added to serum A, 1 mg per l	26 35	23 25	15 10	11 10	25 20	0
7 8 9 10 11	Serum B ₂ from monkey treated with paludrine, pure —ditto—pure —ditto—diluted 1 in 4 —ditto—diluted 1 in 16 —ditto—diluted 1 in 64	42 54 48 28 69	18 28 40 48 22	6 4 8 4 3	4 0 0 0	0 0 0 0	30 14 4 20 6
12 13 14	Liver extract added, pure —ditto—diluted 1 in 4 —ditto—diluted 1 in 16	23 15 35	42 25 20	15 9 15	7 6 10	13 12 20	0 33 0
15 16 17 18 19	Paludrine + liver extract, pure (2 mg per 1) —ditto—pure (2 mg per 1) —ditto—diluted 1 in 4 (0 5 mg per 1) —ditto—diluted 1 in 16 (0 125 mg per 1) —ditto—diluted 1 in 64 (0 03 mg per 1)	4 10 52 48 36	6 0 22 24 24 24	0 0 6 4 16	0 0 4 0 8	2 0 2 0 16	88 90 14 24 0

A sample of serum (B1) was collected from another monkey (B) This served as a control to exclude non-specific antimalarial activity Monkey B was then treated with paludrine, receiving usually two intramuscular injections of 50 mg for a 3 kg animal at 5 pm and 930 am, two hours after the last injection blood was collected from a vein and defibrinated, this provided serum B₂ for the cultures In the experiments with liver extract paludrine was incubated with minced liver exactly as described above The concentration of paludrine used, however, was 14 mg per litre, so that when 01 cc was added to 06 cc medium in the culture tube the final theoretical concentration would be 2 mg per litre tubes to which paludrine was added the paludrine was dissolved in Ringer's solution and added in place of the normal Ringer's solution (01 cc) in the control tubes Precautions to maintain sterility were observed throughout. The tubes were incubated at 37° C After 20 and 42 hours a small sample of the corpuscles was removed by a pipette and smeared out to form thin blood films which were fixed in alcohol and stained with The parasites were examined and Giemsa classified according to the number of pieces of chromatin which they contained, the identity of the slide being unknown to the investigator during examination

The protocol of a typical experiment is reproduced in Table III, which shows

- (1) That many of the parasites develop normally (as shown by the division of chromatin) in the presence of serum from the monkey which supplied the parasitized corpuscles (tubes 1 and 2), serum from a second monkey (tubes 3 and 4), or liver extract (tubes 12–14)
- (2) That the development of the parasites is not prevented by the addition of paludrine to the cultures sufficient to produce a concentration of 1 or 2 mg per litre (tubes 5 and 6), in other experiments the parasites developed normally in a concentration of 20 mg paludrine per litre
- (3) That the development of the parasites is almost completely prevented by serum from a monkey which has received paludrine during the previous 20 hours; this serum is active even when diluted 64 times (tubes 7–11) The parasites in the tubes containing activated paludrine showed various degeneration changes which are described below
- (4) That the development of parasites is similarly prevented by the addition of paludrine

which has been incubated with liver (tubes 15 and 18), although the addition of liver extract alone does not inhibit development (tubes 12-14) Inhibition occurs when the paludrine + liver extract is diluted 16 times (tube 18, paludrine theoretically 0 12 mg per litre) but not when diluted 64 times (tube 19, paludrine theoretically 0 03 mg per litre)

After these experiments had been completed chemical estimations were made of the concentration of paludrine present in the serum of three monkeys which had been previously treated with paludrine, each receiving 50 mg per monkey 18 hours and 2 hours before the serum was obtained The results obtained, using a modification of the method of Spinks and Tottey (1946), are shown in Table IV

TABLE IV

Monkey	Weight (kg)	Dose (mg per kg)	Blood paludrine (mg per litre)
1	5 8	8 6	20
2	2 5	20 0	34
3	3 6	13 9	12

The average concentration of paludrine in the serum of a monkey receiving 50 mg of paludrine as above was thus 22 ± 10.7 mg per litre. This level is approximately equal to the highest concentration of paludrine (20 mg per litre) tested in the control tubes during this work, as recorded above, 20 mg per litre of paludrine solution did not affect the normal development of the parasites. The medium in Tube 11 of Table III was fully active in preventing the development of the parasites although it contained only 1 part of paludrine serum in 64—1 e, probably about 0.3 mg paludrine per litre

The conclusions derived from this work are similar to those derived from the experiments with the tissue cultures of *P gallinaceum*, viz that paludrine itself (even in a concentration of 20 mg per litre) is not active in preventing the development of the parasites *in vitro* but that it becomes active when exposed to the cells of the body (as in the treated monkey) or to minced liver tissue

In a further experiment the supernatant fluid from the incubated tube of paludrine plus liver tissue was heated to 100° C for 20 minutes in order to precipitate all the protein, which was removed by filtration. This filtrate was as active as the unheated supernatant, which indicates that the active derivative of paludrine is probably not bound to protein, and is probably not thermolabile.

Attempts were made to develop a simple technique for testing the activity of paludrine derivatives in vitro by incubating blood containing endo-erythrocytic forms of P gallinaceum with paludrine serum or paludrine + liver extract for several hours and then injecting it into chickens to test for infectivity. The results were very irregular, and most of the specimens were able to infect chickens in spite of exposure to paludrine or its hypothetical products. Accordingly these attempts were abandoned

Morphological description of degenerate parasites

Examination of preparations of *P* cynomolgi from cultures in which the parasites had been subjected to the action of activated paludrine showed that, in addition to a failure of development to mature schizonts, the parasites suffered a series of degenerative changes

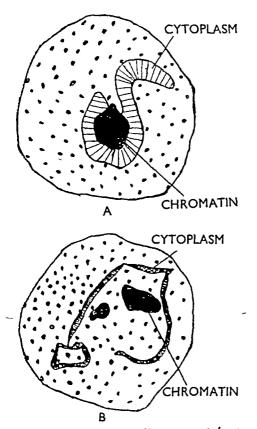


Fig. 1—Malaria parasites (P conomolgi) showing degeneration produced by paludrine serum. A The chromatin is small and dark and the cytoplasm is thinned out and translucent. B The chromatin is condensed into two dark masses, the cytoplasm is disrupted into narrow strands scattered throughout the corpuscle. Magnification about 8,000

A few of the parasites were small, containing one piece of very darkly staining chromatin and having a minimum of cytoplasm, also darkly stained, closely packed round the chromatin Others showed the same small mass of dense chromatin, but had a varying amount of poorly stained pinkish cytoplasm thinned out and almost translucent, so that it was difficult to differentiate from the surrounding corpuscle An example of this type of parasite is illustrated in Fig 1A this particular specimen the cytoplasm was thin and translucent, but not so distended as in others, in which, indeed, the cytoplasm occupied almost the whole corpuscle Many parasites, however, exhibited a more characteristic series of degenerative changes than those just described example is shown in Fig 1B The chromatin was condensed into one or more very darkly stain ing lumps The cytoplasm was disrupted, and was often arranged in narrow dark-staining strands, which were scattered throughout the corpuscles and woven sometimes into fantastic patterns, frequently the strands were completely separated from the chromatin masses, which were left denuded of any cytoplasmic covering This series of changes was more common in samples withdrawn from culture tubes after incubation for 42 hours than in samples withdrawn after only 20 The parasitized red blood corpuscles showed the stippling usual with P cynomolgi This series of degenerative changes infections observed in vitro may be compared with the changes undergone by P vivax when subjected to the action of paludrine in vivo (Mackerras and Ercole, 1947)

DISCUSSION

The experiments described have shown that paludrine in a concentration of 20 mg per litre has no apparent action in vitro on endo-erythrocytic forms of P cynomolgi, similarly a concentration of 2 mg per litre has no action on the exo-erythrocytic forms of P gallinaceum however, paludrine has been previously exposed to the action of body cells, either by injecting it into a monkey or fowl and collecting the serum, or by incubating it with minced rat liver, marked antimalarial action can be demonstrated The simplest explanation for these facts is that paludrine undergoes some chemical modification which converts it into an active compound This hypothesis would explain the contradictory findings of Tonkin (1946) that paludrine had no antimalarial action when added to tissue cultures of exo-erythrocytic forms of P gallinaceum, and of Black (1946), who reported that serum from a patient treated with

paludrine arrested the development *in vitro* of trophozoites of *P falciparum* Marshall (1947) has reported that paludrine solutions are active in inhibiting the uptake of oxygen by *P gallinaceum in vitro*, but the concentrations of paludrine required to produce this effect were 10⁴ to 10⁵ times as great as those used in our experiments (1 in 3,000 to 1 in 6,000 as compared with 1 in 1,000,000 to 1 in 10,000,000) No work has yet been reported on the corresponding activity of paludrine after activation by the living animal or by liver extract

A similar conversion to an active substance occurs with tryparsamide and other pentavalent arsenicals which are converted by cells from the inactive pentavalent to the active trivalent state With paludrine it is not easy to suggest the nature of the hypothetical chemical modi-According to Acheson, King, and Spensley (1947) it is unlikely that activation depends on the conversion of paludrine into a benziminazole Our present information indicates that this activation can be carried out by the whole organism or by minced liver, it cannot be produced by the red blood corpuscles or by the macrophages and fibroblasts present in tissue cultures from the spleen. The further study of the activation of paludrine requires a simpler and more convenient technique for measuring the antimalarial action of compounds in vitro methods used in the present work, although adequate to demonstrate the occurrence of activation, are rather laborious and insensitive for use in a detailed investigation of the phenomenon

The conversion of paludrine by cells into an active derivative is of importance in the study of the action of paludrine *in vitro*, the relation of chemical structure to activity, the search for more active antimalarial compounds, the interpretation of chemical estimations of the blood concentra-

tions of paludrine, and other similar questions, most of this is obvious and does not require elaboration

SUMMARY

- 1 No demonstrable antimalarial action is exerted in vitro on cultures of the exo-erythrocytic forms of *Plasmodium gallinaceum* by paludrine in concentrations of 2 mg per litre, or on endo-erythrocytic forms of *P cynomolgi* by paludrine in concentrations up to 20 mg per litre, these concentrations are higher than those which are commonly reached in the blood during human therapy
- 2 Serum from a fowl or monkey, which has recently received paludrine, exercises a pronounced action on these cultures, preventing the development of the parasites, cultures of *P gallinaceum* are destroyed
- 3 Paludrine which has been incubated with minced liver has a similar pronounced antiplasmodial action in vitro
- 4 These phenomena suggest that paludrine itself is not active against plasmodia but that it can somehow be modified by the body or by liver cells, so that it becomes actively plasmodicidal

Our thanks are due to Mr E C England and Miss J Barten for technical assistance

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A COMPARISON OF THE IRRITANT ACTION OF CONESSINE, ISOCONESSINE, AND NEOCONESSINE

BY

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Conessine is a powerful local anaesthetic, but its use is limited since its injection produces inflammation which may be followed by necrosis *Iso*-conessine and *neo*conessine are isomers of conessine and they also possess local anaesthetic actions. It was therefore of interest to compare their irritant action with that of conessine

No method for comparing the irritant action of different substances in animals has hitherto been described and a method has been used which is in some respects similar to the guinea-pig skin test for diphtheria antitoxin and for old tuberculin In a preliminary experiment 0.2 ml of 1 in 50, 1 in 100, 1 in 200, 1 in 400, and 1 in 800 solutions of conessine dihydrochloride were injected intracutaneously into a guinea-pig and the weals produced by the injection were ringed with ink During the next three days no effect was apparent at the site of injection of the 1 in 800 solution, while the 1 in 400 solution produced only a very slight inflammation The higher concentrations produced progressively greater effects and at 72 hours the site of the injection of the 1 in 50 solution was covered by The fact that these concentrations produced an appreciable and graded response indicated that a comparison could be made in this way

In order to compare the effects produced by the different substances, guinea-pigs were prepared by removing the hair from the back with electric clippers. The back was marked out into 6 areas, 3 on each side of the spine, and 0.2 ml of each of 3 concentrations of one alkaloid (1 in 50, 1 in 100, and 1 in 200) were injected on one side and the same concentrations of a different alkaloid on the other side. In half the animals the solutions were injected in increasing strength towards the head and in the other half this order was reversed. Each concentration of each alkaloid was injected into 6 animals. Inflammation appeared within about 2 hours and a first attempt to compare the effects was made between 2-4½ hours after the injection.

Scores from 0 to 3 were awarded according to the amount of inflammation. After 24 hours the inflammation was more marked and scar tissue was beginning to appear. Estimates of the effects were again made, the range of scores being in creased in accordance with the increased effect After 72 hours the inflammation had largely disappeared and was replaced by scar. Scores from 0 to 6 were again given to the different areas. Two other observers also examined the areas and allotted scores to each. Their results were similar to those already obtained.

The mean result for each concentration of each alkaloid was plotted as an ordinate against the concentration as abscissa (using a logarithmic scale) in Fig. 1, in which the relation between the alkaloids

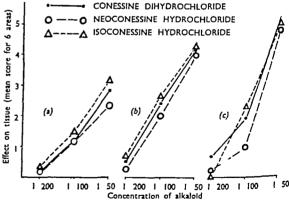


FIG 1—Graphs showing the estimates made by one person of the amount of inflammation and necrosis after the injection of varying concentrations of concessine and its isomers (a) 2-4½ hours after injection, (b) 24 hours, (c) 72 hours

hours, and (c) 72 hours after injection Fig 1 shows that neoconessine causes slightly less inflammatory reaction than the others, but the difference is very small All three substances begin to pro-

duce inflammation in a concentration of 1 in 200. The smallest concentration producing local anaesthetic action is in the range 1 in 6,400 to 1 in 3,200 (Stephenson, 1948), this, however, is a very slight degree of anaesthesia. More is obtained in the range 1 in 1,600 to 1 in 800, which is still well below the concentration causing inflammation.

Solutions containing adrenaline—Procaine is commonly mixed with adrenaline, and its local anaesthetic action is greatly increased thereby. It seemed possible that the local anaesthetic action of conessine might also be increased if adrenaline were mixed with it, and a comparison was therefore carried out in which solutions of conessine were compared with similar solutions containing adrenaline. Six guinea-pigs were used, and the results obtained by the method of Bulbring and Wajda (1945) are shown in Table I

TABLE I

0 2 ml solution is injected intradermally. The aréa is pricked 6 times at 5, 10, 15, 20, 25, and 30 min after injection. The number of times there is failure to respond is observed and is shown below. The maximum number of failures is 36.

Pig No	C	Conessine		Conessine + Adr 1 in 100,000			
	1 in 830	1 in 1660	1 in 3320	1 in 830	1 in 1660	1 in 3320	
1 2 3 4 5 6	36 21 29 35 35 29	25 20 26 22 26 20	15 10 27 15 26 0	35 29 33 35 36 35	34 27 20 28 28 32	30 20 12 15 21 25	
Mean	31	23 1	15 5	33 8	28 1	20 4	

Table I shows that the presence of adrenaline caused some increase in the duration of anaesthesia produced by conessine, but less than that with procaine The difference can be expressed by saying that the addition of adrenaline would enable a solution containing 1 in 1,000 conessine dihydro-

chloride to act like a solution confaining 1 in 700

In view of the fact that the work of R D Haworth and Nazar Singh (1948) indicates that conessine is a steroid, it may well be that it is less readily absorbed from the site of injection than procaine. Thus the fact that adrenaline does not so greatly intensify its local anaesthetic action within the period of 30 min observation is not surprising.

Experiments were also made to observe whether the addition of adrenaline to a solution of conessine would alter the irritant action of the solu-Solutions of conessine dihydrochloride containing adrenaline 1 in 100,000 were compared with the same solution, without adrenaline conessine solutions were 1 in 400, 1 in 200, and 1 in 100, each solution was injected on one side of the back of a guinea-pig and compared with the same solution containing adrenaline on the other Two animals were used, and in neither was any difference observed owing to the presence of The 1 in 100 solution produced obvious inflammation, followed by necrosis 1 in 200 solution produced less inflammation and an induration at the site of injection

SUMMARY

A method of making a quantitative comparison of two or more substances for local irritant action is described. Conessine, isoconessine and neoconessine all begin to cause inflammation after intracutaneous injection in a concentration of 1 in 200. The local anaesthetic action of conessine is intensified when adrenaline (1 in 100,000) is present by about 40 per cent. The presence of adrenaline does not affect the irritant action of conessine.

This work was done when one of us (R P S) was in receipt of a grant from the Therapeutic Research Corporation

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A MODIFICATION OF THE METHOD OF DALE AND LAIDLAW FOR STANDARDIZATION FOF POSTERIOR PITUITARY EXTRACT

BY

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(Received June 28 1948)

The activity of a posterior pituitary extract is still generally determined by the method introduced by Dale and Laidlaw (1912). They used the uterus of a virgin guinea-pig, and the assay consisted in setting limits to the potency of the test solution in terms of the standard solution by the usual ABBA grouping of doses. The method suffered from three defects

- 1 Suitable guinea-pigs were relatively scarce
- 2 The assay often required many hours
- 3 The error of the method was about 20 per cent (Gaddum, 1938)

A different method of assay was described by Thompson (1944) He used the relationship between the dose of pituitary and the fall in blood pressure in the chicken which had been investigated by Smith and Vos (1943) Thompson obtained results with very small errors and was able to assess the reliability of each result from the experimental data He claimed that the method was rapid and easy to perform There is, however, one drawback to the method It is possible that the active principle responsible for the fall in blood pressure is not identical with that which makes the uterus contract Thompson himself found a significant difference between potency on the blood pressure and potency on the guineapig uterus in 1 out of 17 parallel determinations and suggested that the blood pressure method cannot always be relied upon to estimate the oxytocic activity

It seemed best, therefore, to return to the original method of standardization using the isolated uterus as a test preparation. Various modifications of the original method have recently been introduced. Morrell, Allmark, and Bachinski

(1940) used 8 strips of muscle which were cut from the same uterus and suspended in the bath together They showed that the percentage of strips responding was related to the log of a dose of pituitary put into the bath By this measurement of "all or nothing" responses Morrell et al obtained more accurate estimations of oxytocic activity than previous workers, but they made no statement of the reliability of each estimate One estimate of potency had an error of 35 per cent If this was associated with a high standard error for that particular determination the observer would have been warned-that the estimate was unreliable, but it was included in a table of results all of which were considered satisfactory. As Bliss (1941) pointed out, "a determination of potency should always include an estimate of its error, computed as an integral part of the assay" Gaddum (1933) provided the basis for a biological assay which included an estimate of the precision of the result and showed that his formulae applied equally well to measured responses and to quantal data Schild (1942) published an account of a null hypothesis assay conducted on statistically sound principles His method has been followed almost completely to obtain the results in this paper

METHODS

A modification of the classical method of Dale and Laidlaw was adopted

The rats uterus was used as test preparation since rats are cheaper and more easily obtained than guineapigs. Moreover, Garcia de Jalon, Bayo Bayo, and Garcia de Jalon (1945) have shown that most rats uten do not contract spontaneously in Locke's solution in which the calcium and glucose concentrations are 1 and 1 respectively of the usual. One hom of the uterus was suspended in a 10 ml bath in modified Locke's solution. One end was attached to an

The lever was so arranged that the load on the uterus was about 1.2 g and the contractions were magnified four times. It was equipped with a glass frontal writing point and its angular excursion was limited to 30° each side of the horizontal

The bath was supplied with oxygen containing 5 per cent CO_2 , this was to eliminate possible changes of pH, but the CO_2 is probably unnecessary when the Locke's solution is replaced at short intervals

Non-pregnant white rats were used Their weights varied from 120 to 200 g No record was kept of the position in the oestrus cycle of each rat Table I shows the variation which may be expected within a group of rats

TABLE I

Assay No	Spontaneous contractions	Remarks
1, 2, 3, 9 10 5, 6, 7 4 8	No Yes, overcome Yes, overcome Yes Yes	Satisfactory Sassays Unsatisfactory assays Useless

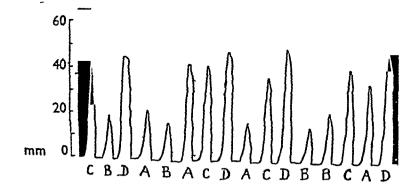
It will be seen that 6 rats were good, 2 poor, and 1 useless out of a total of 9 In practice the rats in assays 4 and 8 would have been discarded after a short time. The uterus in assay 4 produced only 12 contractions. In assay 8 the uterus had so much spontaneous activity that it was obviously unreliable.

Performance of an assay

- 1 The rat is killed by a blow on the head and bled out. The uterus is removed and one horn is suspended in the bath and attached to the lever. The temperature must be constant at about 32° C.
- 2 Doses of pituitary should be given without delay at regular intervals of 3 or 4 min. Two doses must be found such that the contraction for the higher dose is at least twice as great as that for the lower dose. Dose ratios of 4 3, 3 2, 8 5, and 2 1 have been used (4 3 is the most common). In order to ensure linearity between response and log dose it is best to use contractions below 80 per cent of maximal (see Tests for Linearity below)

If spontaneous contractions are troublesome they may sometimes be overcome by lowering the temperature or by reducing the time between successive doses If the uterus is very insensitive (i.e., will not respond to 0.05 unit) the sensitivity may sometimes be increased by raising the temperature

- 3 The strength of the unknown must be guessed by matching it with the standard. The error of the assay is much smaller if a good guess is made.
- 4 The assay is now continued exactly as described by Schild (1942) for histamine assays. Four doses are used, two of standard and two of unknown. The ratio of high dose to low dose should be the same for standard and unknown. Each dose is given once in each group of four doses and its position within the group is decided by chance. Fig. 1 shows the record obtained in one assay. The drum was turned on 15 sec before the dose was given. The uterus



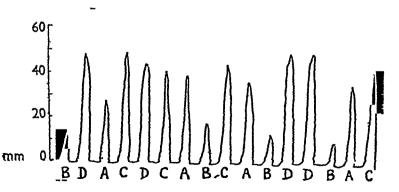


FIG 1—An assay of pituitary The record shows thirty-two contractions of a rat's uterus The contractions are responses to four different doses of pituitary A, B, C, and D each of which is given once in each group of 4 contractions There are eight groups in all A = 0.05 units, B = 0.04 units, C = 0.064 units, and D = 0.08 units B and C were treated as "standard" A and D were treated as "unknown" A D = B C = 5.8 Estimate of unknown standard = 1.25

True value unknown standard = $\frac{3000}{0.04}$ = 1.25 A dose was put into the bath every 4 min and washed out after 45 sec. Weight of rat 140 g. Temperature 34°-36° C. Load on uterus 1.3 g. Total experimental time 4 hr.

contracted for 45 sec, after which the drum was stopped and the bath was refilled with Locke's solution A and D were dilutions of the unknown, and B and C were dilutions of the standard, C was stronger than B in the proportion 8 to 5 The ratio of D to A is the same as that of C to B

TABLE II Heights of contractions in mm = y

	Dose units	log dose = x	1	2	3	4	5	6	7	8	Sum
D C A B	0 064	0 806 0 602	48 43 21 21	50 43 43 18	52 38 17 16	51 42 35 25	49 48 28 16	45 43 39 19	52 44 37 14	51 44 35 12	398 345 255 141
	oup tot	1		154							1139

RESULTS

The results of this assay are shown in Table II In Fig 2 the mean height of contraction in response to each dose is plotted against the log

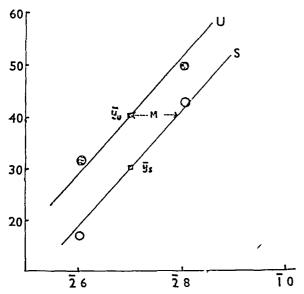


Fig 2—Ordinates mean height of contraction in mm Abscissae \log_{10} dose in units of pituitary. White circles are the mean responses for high and low doses of standard, black circles are the mean responses for high and low doses of unknown. The black squares $\bar{\jmath}$ and $\bar{\imath}_{u}$ are the means of all the responses to standard and unknown. S and U are the regression lines relating response to \log dose of standard and unknown. The lines were drawn by eye. M is the distance between the lines. $M = \log$ unknown— \log standard $= \log \frac{\text{unknown}}{\text{standard}}$. Graphically M = 0.09

$$\frac{\text{unknown}}{\text{standard}} = \text{antilog } 0.09 = 1.23$$

The "null hypothesis" states that there is no difference between the standard solution and the unknown solution, and so the standard log doses are used in plotting the points for the unknown Since the unknown and standard contained the same active principle the two lines joining the mean responses to high and low doses of each are parallel The lines are the regression lines relating height of contraction to log dose, and they were drawn by eye to be the parallel lines that fitted the points most nearly The regression coefficient b is the slope of each line. An estimate of the potency of the unknown solution can be made by using the two regression lines The horizontal distance (M) between the lines is the difference between log doses producing the same effect, and it is therefore the log of the ratio of unknown to standard By elementary geometry

$$M = \frac{\bar{y}_u - \bar{y}_s}{h}$$

where \bar{y}_u and \bar{y}_s are the mean responses to unknown and standard respectively, if b is known M may be calculated

An estimate of potency of a preparation does not give much information about the true potency unless it is accompanied by a statement of its The simple graphical measurement of M as described above does not enable us to estimate the reliability of the estimate M, for this we need to calculate the standard error of M In order to do this M should be calculated rather than determined graphically, and its standard error must also The methods of calculation and be calculated analysis of variance given by Schild are simple and An example is given below When the method of assay is slightly varied, for instance, to include three dose levels of standard and of unknown, more complex methods must be used These may be found in any standard textbook of statistics, but are given particularly clearly by Finney (1947)

Calculation

In order to determine M we must calculate the numerator and denominator of the expression

From Table II
$$\tilde{v}_u = \frac{398 + 255}{\frac{1}{2}n}$$
 and $\tilde{v}_s = \frac{345 + 141}{\frac{1}{2}n}$

where n is the total number of responses, n=32

2 When only 4 doses are used the equation for b is

$$b = \frac{\text{sum of responses to high doses} - \text{sum of responses to low doses}}{\frac{1}{2}n \times (\log \text{ high dose} - \log \text{ low dose})}$$

With the data from Table II

$$b = \frac{(398 + 345) - (255 + 141)}{\frac{1}{2}n \times d}$$
where $d = \log \text{ high dose} - \log \text{ low dose}$

$$= \log 0.064 - \log 0.04$$

$$= \overline{2}.806 - \overline{2}.602$$

$$= 0.204$$

The value of b will be needed later It is $b = \frac{398 + 345 - 255 - 141}{16 \times 0204}$ = 1063

For calculation of M, however, it is simpler to use the above expressions, noticing that $\frac{1}{2}n$ occurs in numerator and denominator of the express on for M and so disappears

$$M = \frac{[(398 + 255) - (345 + 141)] \times 0204}{(398 + 345) - (255 + 141)}$$
$$= \frac{167 \times 0204}{347}$$
$$= 0.09811$$
$$\frac{Unknown}{Standard} = R = ant log M = 125$$

Since this assay was conducted with two dilutions, "unknown" and "standard," of the same pituitary extract, the true value of the ratio $\frac{\text{"unknown"}}{\text{standard}} = \rho \text{ was known The "unknown" doses of D}$ and A were 0 08 unit and 0 05 unit respectively Hence

$$\rho = \frac{D}{C} = \frac{A}{B} = \frac{0.08}{0.064} = 1.25$$

In order to make a statement about the reliability of the estimate R, the standard error (s_M) of M must be calculated. This is obtained from the standard deviation (s_y) of a single observation (y). The standard deviation of a single observation—the error of the assay—can only be obtained through an analysis of variance which segregates variation due to known causes from that due to the variability of the preparation

Table III shows that the sums of squares of deviations attributable to the variation

1 between groups (variation of the preparation with time)

$$= \frac{1}{4}(133^2 + 154^2 + 123^2 + \text{etc}) - \frac{1139^2}{32} = 182$$

2 between standard and unknown
=
$$\frac{(398 + 255 - 345 - 141)^2}{32} = 872$$

3 between high doses and low doses (regression)

$$= \frac{(398 - 345 - 255 - 141)^2}{32} = 3763$$

4 between the slopes of the two regression lines (deviations from parallelism)

$$=\frac{(398+141-345-255)^2}{32}=116$$

The sum of squares of deviations of all the observations from the common mean = total sum of squares

$$= (48^{2} + 50^{2} + 52^{2} + \text{ etc}) - \frac{1139^{2}}{32}$$
$$= 5536$$

When all the sums of squares due to known causes have been subtracted from the total the remainder is due to the error of the assay. This remainder, divided by its degrees of freedom, gives the variance of a single observation according to the usual formula for standard deviation. The standard deviation of a single observation is the square root of the variance. In the example the residual sum of squares of deviations = 5536 - 182 - 872 - 3763 - 116 = 603. This had 21 degrees of freedom because from the total number (32-1) 7 must be subtracted for the 8 different groups and one each for the other three known sources of variation.

The variance
$$s_y^2 = \frac{603}{21} = 2871$$

TABLE III

Source of variation	Sum of squares	Degrees of free- dom	Varı- ance	F	P
1 Groups 2 Standard an	182	7	26	11	>0 0 5
unknown	872	1	872	30	< 0 01
3 Regression	3763	1	3763	131	< 0 01
4 Deviation from				}	
parallelism	116	1 1	116	4	>0 05
5 Error	603	21	28 71	,	
Total	5536	31			

F is the variance ratio, e.g., for "Deviation from parallelism" $F = \frac{116}{287} = 4$ A table of F for $n_1 = 1$ and $n_2 = 21$ (where n_1 , n_2 are the degrees of freedom for "Deviations from parallelism" and for "Error" variance respectively) gives P > 0.05 This means that there is a proba-

bility of more than 5 per cent that this deviation from parallelism would occur by chance and so the slopes of the two regression lines are not significantly different. The values given in the column P are the probabilities that such variation would occur by chance. Thus there is less than a one pericent probability that so great a variation between standard and unknown would have occurred had the unknown not been different from the standard. The unknown is said to be significantly different from the standard Similarly, the regression is significantly different from zero. The variation between groups is not greater than would be expected by chance in more

than one in twenty experiments (Here
$$F = \frac{287}{26}$$

= 11 because
$$F = \frac{\text{larger mean square}}{\text{smaller mean square}}$$
 and hence $n_1 = 21$ and $n_2 = 7$)

The standard error of M

The standard error of the estimate of M may be obtained from Schild's formula

$$s_{M}^{2} = \frac{4s_{V}^{2}}{nb^{2}} \left(\frac{M^{2}}{d^{2}} + 1 \right)$$

In the example

$$s_{M}^{2} = \frac{4 \times 2871}{32 \times (1063)^{2}} \left[\frac{(0.09811)^{2}}{(0.2041)} + 1 \right]$$

$$= \frac{114.84 \times 1.2312}{361600}$$

$$= 0.0003909$$

$$s_{M} = \sqrt{0.0003909} = 0.01976$$

The fiducial limits of M

The limits of the value of M are obtained from adding and subtracting the standard error of M multiplied by t. The value of t depends on the degrees of freedom associated with the error sum of squares. In the example there were 21 degrees of freedom, and so t is obtained from the table under n=21. For a probability level of 0.05, $t_{21}=2.08$

$$s_{x} \times 2080 = 00421$$

$$M \pm s_{\rm M} \times 2080 = 00981 \pm 00421$$

Hence the fiducial limits (P = 0.05) for M are 0.0560 and 0.1402

The limits for R are

$$R_1 = \text{antilog } 0.0560 = 1.14$$

 $R_2 = \text{antilog } 0.1402 = 1.38$

That is to say, there is only one chance in twenty that these results would have been obtained if the true value of the unknown lay outside the limits 114 - 138

Now 1 38 - 1 14 = 0 24 and
$$\frac{0.24}{2}$$
 = 0 12

the approximate range of results is $R \pm 0.12 = 1.25 \pm 0.12$

Hence the limit of error is
$$\frac{0.12}{1.25} \times 100 = 9.6\%$$

(This assumes that R_1 and R_2 are equidistant from R, which is not strictly true since M_1 and M are equidistant from M, which is on a logarithmic scale, but the assumption is true enough for an approximate calculation of the limits of error)

TABLE IV

Assay	Time	n	ρ	R	R_1	R,	E	s _y /b
1 2 3 4 5 6 7 8 9	5 4½ 5 2 4 3½ 3 8 2½ 2	36 32 32 12 24 24 24 24(-4) 20 20	1 25 1 25 1 14 0 80 1 08 1 07 1 07 0 91 1 00 1 07	1 22 1 25 1 10 0 91 1 10 1 09 1 05 1 06 0 99 1 01	0 97 1 14- 1 06 0 81 0 96 1 02 0 96 0 77 0 94 0 96	1 55 1 38 1 13 1 02 1 25 1 18 1 15 1 56 1 04 1 06	2 6 0 3 6 14 1 9 1 25 1.25 15 0 85 5 8	0 145 0 050 0 018 0 044 0 062 0 034 0 044 0 131 0 022 0 022

Table IV includes all of a recent series of 10 assays in order to show the variation which may be expected from a group of 9 rats. Eight out of these 10 assays were considered satisfactory. Two assays (4 and 8) were unsatisfactory and in practice the results from them would have been subjected to further tests. The uterus gave only 12 contractions in assay 4 and then lost all sensitivity. In assay 8 the uterus had a great deal of spontaneous activity and the contractions were obviously unreliable. The assays which are bracketed were performed on the two horns of the uterus from the same rat

In assays 4–10 the true value of the ratio of unknown to standard (ρ) was unknown to the observer until after the results were obtained. The columns R, R_1 and R_2 show the estimate of ρ and the two fiducial limits (R_1 and R) calculated as described above for P=0.05. It will be noticed that ρ falls within the range R_1-R_2 except in assays 3, 4, and 10, where it is just outside the range Recalculation of R_1 and R_2 according to Schild's equation for the exact limits, which is given in a footnote to his paper, did not alter the values. It must be borne in mind, however, that the statistical analysis is based on the assumption that all measurements are absolutely

accurate and the calculation of error does not include unavoidable errors in pipetting which occur during the dilution of the test solutions. Hence, in order to make a reliable statement about the potency of an unknown, the fiducial limits should be extended by 4 per cent (this allows for 1 per cent error for each dilution). The figures in the column "Time" are the times in hours required for the experimental part of each assay and E is the actual percentage error in the estimate of potency

The ratio of the standard deviation of a single response to the slope of the regression line is shown in the last column of Table IV (s_y/b) It is an inverse measure of the usefulness of the preparation and for a satisfactory assay (s_y/b) should not be much greater than 005

Gaddum s calculation

Gaddum (1938) calculated the standard error of pituitary assays performed by the method of Dale and Laidlaw. He used only those assays which were considered satisfactory and obtained a value for the standard error of 7 73 per cent, which gave fiducial limits at 19 9 per cent (P=0.01). If his calculation is repeated with the results of the assays in Table IV, excluding assays 4 and 8 for reasons given above and using ρ instead of his mean estimated potency, the standard error is 2.84 per cent and fiducial limits are at 9.4 per cent (for P=0.01 $t_7=3.499$), which is about half the value found by Gaddum for the original Dale and Laidlaw method

The dose-response relationship

The calculations and statistical analysis of the results are based on the assumption that the relation between response and log dose is exactly linear. In fact, the curve relating response to log dose is sigmoid and approximates to linearity over part of the response range. Clearly it should be the aim of the observer to confine the responses of the uterus to this linear part of the range. Since the region of linearity varies from one preparation to another it must be left to the observer to decide its position, but it has been found that for most uteri any responses less than 80 per cent of the maximal can be used.

Tests for linearity

Assay No 1 was exceptional in that 6 different doses (3 standard and 3 unknown) were used Here a test of linearity was possible without knowing the true value of the "unknown" It showed that there was no significant departure from linearity. The calculations for this assay were

slightly more complex than for the 4-dose assays and were performed as described by Finney (1947). The 6-dose design is not recommended for routine work because the experimental procedure is more complicated and the time interval between successive doses may be too short for the additional diluting needed for 6 doses.

It should be realized, however, that the 4-dose design suffers from the defect that no test of linearity can be applied from the data of a single assay. The worker is safeguarded to a certain extent by the test for parallelism. The variance due to deviations from parallelism would be significant if there were any serious departure from linearity and if M were not negligible

Table V shows the variance ratios (F) for deviations from parallelism. An F value smaller than that for P=0.05 indicates that the deviation was not significant. In assay 9 the deviation from parallelism was just significant. In this assay the strengths of the unknown and standard were

TABLE V

Assay	Devia- tion from parallel- ism F	F(P = 0 05)	F(P = 0 01)	Highest contrac- tion mm	Lowest contrac- tion mm
1 2 3 4 5 6 7 8 9	26 14 10 34 10 1 5 2 1 5 5 2 50	249 244 244 249 244 244 4 3 4 75 4 75 244	9 33	65 52 73 61 32 35 49 85 80 96	2 16 3 15 11 1 0 17

exactly the same owing to a lucky initial guess of the potency Hence it was not possible to calculate the deviation from linearity as was done by Schild However, the estimate of the potency of the "unknown" in assay 9 was a good one, which is in accordance with Schild's conclusion that the method is relatively insensitive to deviations from parallelism The last two columns in Table V show the highest and the lowest contractions as recorded on the drum The maximum contraction was generally not recorded, but general experience with the rat uterus preparation indicates that most uters are capable of 100 mm contraction with the same apparatus, and so the figures in the table are approximate percentages of the maximum

SUMMARY

- 1 Schild's null hypothesis method was applied to the assay of posterior pituitary extract according to Dale and Laidlaw
- 2 A rat's uterus was used as the test prepara-
- 3 One assay is described and the result is calculated by Schild's method
- 4 Six rats out of a total of nine could be used for satisfactory assays
- 5 Eight satisfactory assays were performed The mean percentage error was 216 Fiducial limits for the estimate of potency were calculated The mean experimental time of an assay was 3½ hours

This work was done at the suggestion of Prof J H. Burn My thanks are due to Dr D J Finney and his staff for help in the calculations

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SULPHYDRYL ADDITION COMPOUNDS OF SOME QUINONES AND RELATED SUBSTANCES IN THEIR ACTION ON THE GROWTH OF NORMAL CELLS

BY

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We have reported recently (1948) that maleic acid produces in tissue cultures of chick fibroblasts a mitotic inhibition of the same kind and of the same order as 2-methyl-1 4-naphthohydroquinone diphosphate, studied by J S Mitchell and I Simon-Reuss (1947) with the same biological material This similarity is easily understood if the substituted hydroquinone is degraded in the dividing cell to a quinone, as the quinone molecule contains in its aliphatic part the residue The question therefore arises of maleic acid whether maleic acid and the quinones have properties in common which may help us to understand the similarity of their antimitotic activity

Maleic acid and the quinones excel by the ease with which they add other molecules the substances which are added the sulphydryl compounds play a prominent part, physiologically as well as chemically Their role in cell division is well established by the investigations of Shearer (1922), Hammet (1930), Rapkine (1931), Ephrussi (1931), Chalkley (1937), Brachet (1940), and many others Chemically it has been shown that maleic. acid adds thiolacetic acid, cysteine, and glutathione to give well-defined products Furthermore the reactivity of maleic acid towards -SH compounds permits its use as an inhibitor of -SH enzymes (Morgan and Friedmann, 1938a, b, and c) the basis of these results the retardation of malignant growth by maleic acid (Brunschwig et al, 1946) has been discussed The fixation of -SHcontaining enzymes by quinone has been suggested by Potter (1942) as an explanation of the growthinhibiting action of azo dyes. In the naphthoquinone series Fieser and Fieser (1944) emphasize the smooth addition of -SH compounds by naphthoquinones in connection with their physiological

activity Colwell and McCall (1945) suggest that the mode of antibacterial action of 2-methyl-1 4naphthoquinone is a blocking of essential enzymes or essential bacterial metabolites by its combination with sulphydryl groups

In our own experiments (1948) a parallelism between mitotic inhibition and -SH uptake is apparent. Thus maleic acid adds -SH compounds it is a strong antimitotic. The transisomer, fumaric acid, and the methyl derivatives, citraconic acid and mesaconic acid, show no -SH uptake they are devoid of antimitotic activity. Naphthoquinone and its 2-methyl derivative easily add -SH compounds they are both strongly antimitotic.

The parallelism between mitotic inhibition and SH uptake, manifested by maleic acid and the quinones, has been used by us as a starting point for an experimental approach to the underlying problems

As in tissue cultures the diphosphates of the hydroquinones and not the free quinones were applied, a comparison between maleic acid and the quinones presupposes that the quinones have the same degree of antimitotic activity as the hydroquinones Lehmann (1942) has shown that this is so Nevertheless we are fully aware that our choice is an arbitrary one. It compels us to set aside the processes which lead from the diphosphates of hydroquinones to the quinones, reactions which may be associated with physiological processes as important as, if not more important than, those connected with the quinone structure

The naphthoquinones (I) and maleic acid (IV) behave somewhat differently with -SH compounds maleic acid forms saturated thio-ethers

of succinic acid (V), but the corresponding naphthalene derivatives (II) are so easily oxidized by unchanged quinone that only thio-ethers of naphthoquinone (III) can be isolated

Addition products of type (III), where R=H or CH_3 , have been prepared from the quinone and

thiolacetic acid and glutathione. Addition products of type (V) have been prepared from maleic acid and thiolacetic acid, glutathione and cysteine. The action of these substances on the growth of normal cells has been investigated. The present communication gives the results obtained so far

EXPERIMENTAL

The experiments were carried out on tissue cultures of chick fibroblasts The technique used has been described in our first paper (1948), to which we refer for details

The values for mitotic inh.bition and phase distribution obtained with the different substances are collected in the Table The cytological description will be given by Mrs I Simon-Reuss in another paper

1 S-(1 4-naphthoquinonyl-2)-thiolacetic acid (III, R=H, R'= S CH₂CO₂H)

The Table shows that the addition of thiolacetic acid to 1 4-naphthoquinone has abolished the strong antimitotic properties of 1 4-naphthoquinone. The phase distribution has also become normal No abnormal mitoses have been observed

TABLE

Tissue culture—chicken fibroblasts, hanging drop method, 4th passage, 24 hr cultures, fixed in Susa, stained in Heidenham's haematoxylin

		Molar	Mitoses as	Per ent	Pha	ase distribution	ı ıр % of mitc	oses_
)	Exp	conc	% of mitoses of controls	inhibition	Prophase	Metaphase	Anaphase	Telophase
s-(1	-NAPI	THOQUINONYL-	2)-THIOLACETIC ACI	D (5210 mitor	tic cells invest	igate!)		
5	2 3 4 6 7	Controls 1 × 10 ° 3 × 10 ° 5 × 10 ° Controls 4 × 10 ° 6 × 10 °	94 6 ± 5 4% 102.8 ± 9 1% 98 6 ± 8 2% 99 3 ± 2 4% 99 6 ± 2 5%	— — — — —	14 0 13 7 14 5 14 4 15 2 15 6	36 9 43 1 41 6 42 3 27 9 45 1 43 6	89 72 45 17 26 25	40 2 36 7 37 5 38 7 56 0 37 1 38.2
s-(1	4-NAPI	ATHOQUINONYL-2	2)-glutathione (93	90 mitotic cel				
5	2 3 4 6 7 8	Controls 2 × 10 6 4 × 10 6 6 × 10 6 Controls 2 > 10 8 4 × 10-6 6 × 10-6	99 3 ± 7 8 % 100 9 ± 6 4 % 99 8 ± 7 7 % 		16 3 17 6 17 7 17 4 18 9 11 7 16 9 13 4	39 2 36 5 38 8 38 9 22 1 26 3 24 1 26 1	70 81 79 76 60 43 56 53	37 5 37 8 35 6 36 1 53 0 57 7 53 8 55.2
s-(2-	-METHY	L-1 4-NAPHTHO	QUINONYL-3)-THIOLA	CETIC ACID (5	545 mitotic cel	ls investigated)		
1	2. 3 4	Controls 1 × 10 ° 3 × 10 ° 5 × 10 °	92.1 ± 3 4% 99 7 ± 6 0% 110 5 ± 9 8%	=	17.5 17 2 14 4 13 9	27 0 28 1 32 8 32 4	66 59 54 5.2	48 9 48 8 47 4 48 6 40.2
, 5	6	Controls 4 × 10 ° 6 × 10 °	101 4 ± 2.9 % 100 7 ± 2.5 %		14 0 17 7 16 5	36 9 42.9 40 0	8 9 3 7 3 1	35 7 40 4

TABLE (continued)

S-(2-METHYL-1			_	,	TABLE (co				
Exp			Molor		Per cent	Pha	se distribution	in % of mito	ses
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	Ex	ф		% of mitoses of controls		Prophase	Metaphase	Anaphase	Telophase
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	S-(2-M	ETHYL	-1 4- NAPHTHOO	UINONYL-3)-GLUTAT	THIONE (6291	mitotic cells i	nvestigated)	-	
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$		1				174	34.5		43 2
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	•	2	1×10^{-7}		_	91			185
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$			3×10^{-7}	900 1 3 09/	10.1				
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	~ =	4		·	—		350		45 3
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	3	2	2×10^{-6}	441±30%		128	369	4 1	
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $			4×10^{-6}	$470 \pm 15\%$					
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	_	6		$42.6 \pm 2.0\%$	57 4				39.4
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	7	0		115 + 40%	55.5	181			
S-THIOLACETO-SUCCINIC ACID (5539 mitotic cells investigated) r 1	ς.	8 Q	4×10^{-6}	41 2 ± 58%		14 7		3 6	33 7
S-THIOLACETO-SUCCINIC ACID (5539 mitotic cells investigated) r 1			6×10^{-6}	443±34%	55 7	116	49 2	12 1	27 1
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	S_THI	OT ACE	TO-SUCCINIC AC		ells investigate	ed) ^			
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$		OLITOS	_		. —		1 364	2,6	
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	1	2	1 × 10 ⁻⁶	$939 \pm 77\%$		179	38 4		41 6
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$		3	3×10^{-6}	$71.5 \pm 4.8\%$					
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	_	4		84 5 ± 6 8%	15.5	17.5			
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	5	6		960 + 28%	40				52.5
S-CYSTEINO-SUCCINIC ACID (5081 mitotic cells investigated) 1			$\frac{2 \times 10}{4 \times 10^{-6}}$	708 + 24%		190	26 3	23	52 4
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$				663 ± 33%	33 7	18 3	21 9)	58 9
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	S-CY	STEINO	-SUCCINIC ACID	(5081 mitotic cells	investigated)			,	
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$				1	1 –		313	1 3	45 1
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	_	2		$847 \pm 53\%$		199	32 8	06	467
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$		3		$813 \pm 54\%$					
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	_	4		543±34%	45 7				
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	3	6		683 + 71%	31.7			2.5	55 1
S-GLUTATHIONO-SUCCINIC ACID (7964 mitotic cells investigated) 1				683 + 59%	31 7	21 4	24 2	3 1	51 4
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$				$37.5 \pm 3.1\%$	62 5	19 4	165	1 20	62 1
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	S-GI	LUTATE	HONO-SUCCINIC	ACID (7964 mitotic	cells investig	ated)			-
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$				· —	ı —	•	192	84	52 0
5 Controls 149 349 40 456			1×10^{-6}	860 ± 48%					49 7
5 Controls 149 349 40 456			3×10^{-6}	$73.5 \pm 5.5\%$				178	
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	5	4) 34 y ± 4 3 %	45 3	149	34.9	46	428
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	3			701 + 66%	29 9	20 7			
$\begin{array}{c c c c c c c c c c c c c c c c c c c $		7	4 × 10 ⁻⁶	$659 \pm 65\%$	34 1	160	40 6	72	36 1
		8	6×10^{-6}	$55.7 \pm 6.1\%$	44 8	18 9	28 0	5 3	47 6
			<u>. L</u>	<u>l</u>	<u> </u>	1	<u> </u>	<u> </u>	!

² S-(1 4-naphthoquinonyl-2)-glutathione (III), R=H, R'=SG)

The substance gives no mitotic inhibition, the phase distribution is normal. Abnormal cells have not been found

Mitoses and phase distribution are normal

4 $S-(2-methyl-1 \ 4-naphthoquinonyl-3)-gluta-thione (III, <math>R=CH_3$, R'=SG)

The addition of glutathione to 2-methyl-1 4-naphthoquinone gives a product which has definite

antimitotic properties Mitotic inhibition seems to start at 5×10^{-7} M with inhibition of 10 per cent. The mitotic inhibition increases with rising concentration at 2×10^{-6} M it is 56 per cent. This value seems to represent the maximum of mitotic inhibition, as further increases in concentration are not followed by greater mitotic inhibition. The phase distribution shows features of interest. Distributions are distribution have been observed at 1×10^{-7} and at 3×10^{-7} M, i.e. at concentrations where no mitotic inhibitions are found in the 24-hour tissue cultures. Here phase disturbances precede mitotic inhibition. In contrast, no phase

³ S-(2-methyl-1 4-naphthoquinonyl-3)-thiolacetic acid (III, $R=CH_1$, $R'=SCH_2CO_2H$)

disturbances are to be seen in the 24-hour cultures when the mitotic inhibition reaches its peak. The phase disturbances consist in an accumulation of metaphases and anaphases and a loss of telophases

S-(2-Methyl-1 4 Naphthoquinomyl-3-) glutathions Phase distribution in % of total mitoses, compared with mitotic inhibition.



Fig 1—Disturbances in phase distribution preceding mitotic inhibition

(Fig 1) Abnormal mitoses are present in the whole range of concentrations investigated, even at 1×10^{-7} and 3×10^{-7} M

5 S-thiolaceto-succinic acid (V, R=SCH₂CO₂H)

The substance has weak antimitotic properties At 6×10^{8} M the mitotic inhibition is only 33 7 per cent. The phase distribution is apparently not disturbed. Abnormal mitoses are present in all concentrations investigated.

6 S-cysteino-succinic acid (V, R=SCH₂CH (NH₂)CO H)

The mitotic inhibition of S-cysteino-succinic acid is more pronounced than the inhibition by

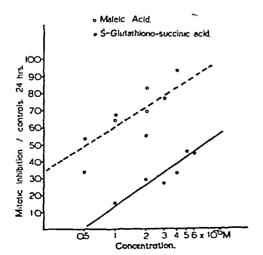


Fig 2.—Parallelism of the concentration curves showing the mitotic inhibition of maleic acid and of S-glutathionosuccinic acid

substance 5 At 6 \times 10 6 M an inhibition of 62.5 per cent has been found. The phase distribution is normal. A few abnormal cells are present at all concentrations. The small outgrowth of the cultures at the higher concentrations (5 and 6 \times 10-6 M) has to be mentioned.

S = glutathiono-succinic acid (V, R=SG)

The mitotic inhibition of S-glutathiono-succinic acid is weaker than the inhibition produced by the cysteine adduct (substance 6). The inhibition increases with rising concentrations. The concentration curve follows a logarithmic line when plotted against the \log_{10} of the concentrations. The graphic comparison with the corresponding line representing the concentration curve of maleic acid shows that the two lines take parallel courses (Fig. 2). The phase distribution shows accumulation of metaphases and irregular anaphases, abnormal mitoses are to be seen at all concentrations

DISCUSSION

The experiments carried out with sulphydryl addition compounds of some quinones and of maleic acid, described in this paper, deal with two groups of adducts which are different in the part of the molecule, apparently connected with their physiological activity. The quinones have given unsaturated addition products (III) which can be regarded as derived from maleic acid, whilst the maleic acid adducts are saturated succinic acid derivatives (V)

Mitotic inhibition has been found in both Unsaturation of the adducts, therefore, does not seem to be the decisive factor determining This result can be suptheir mitotic activities Lettré and ported by an interesting analogy Mohn (1946) have found that the dihydroderivatives of diethyl stilboestrol have antimitotic properties as well as the unsaturated diethyl stilb-On the other hand, v Mollendorff's observations (1941) on male sex hormones show clearly that in other groups the unsaturation of the Only the unsaturated molecule is essential members of this group develop antimitotic properties, whilst the activity as male sex hormones is displayed by the saturated compounds as well The possible physiological transition of the substances investigated by us from saturation to unsaturation or conversely must be kept in mind for further investigations

The addition of SH compounds to maleic acid results in the formation of a centre of asymmetry in the adduct when thiolacetic acid is used for this reaction, or of an additional centre when cysteine or glutathione are used We intend to

supplement our investigations by resolving S—thiolaceto—succinic acid into its optical isomers and investigating their antimitotic activities S—cysteino—succinic acid seems to be formed under the stereochemically directing influence of L—cysteine (Morgan and Friedmann, 1938c)

The results obtained with the sulphydryl addition compounds of 2-methyl-1 4-naphthoquinone are interesting in several respects. The Sthiolaceto-derivative was completely inactive as an inhibitor of mitosis, whilst the glutathione derivative was active Here, clearly, the importance of the sulphydryl-carrying reactant comes to light. It has been stressed from the beginning in the maleic acid series that the reactivity of the sulphydryl group with the activated double bond is different from case to case In the aumone series the interesting results of Walsh and Walsh (1948), showing that liver hexosediphosphatase exhibited a much greater sensitivity towards quinones than did the other phosphatases, point -in the same direction

The activity of the glutathione derivative of 2-methyl-1 4-naphthogumone as an inhibitor of mitosis, compared with the lack of antimitotic activity of the methyl free S - (1 4-naphthoquinolyl-2)-glutathione, shows a new feature of the methyl group So far we have seen that the introduction of a methyl group in the quinones as well as in maleic acid has been followed by a decrease of their antimitotic activity elimination of the methyl group leads to a product which has lost its antimitotic activity Chemical analogy shows that the 2-methyl group exerts a distinct retarding effect in the introduction of substituents at position 3 by way of 1 4 addition reactions (Fieser and Fieser, 1944, pp. 737 and 743), but there are exceptions where the presence of a 2-methyl group increases the reactivity

The adducts of thiolacetic acid and of glutathione to 1 4-naphthoquinone were both inactive as antimitotic agents. Other sulphydryl substituents have not been investigated. The different biological results obtained by adding thiolacetic acid or glutathione to 2-methyl-1 4-naphthoquinone show that the possibility cannot be dismissed that other —SH addition products to 1 4-naphthoquinone may display antimitotic activity

The substances formed by the addition of thiolacetic acid to some quinones are acids in which the sulphur is linked ether-fashion. If one replaces the sulphur by oxygen one comes in the aromatic series to substances related to phenoxyacetic acid. These are known to be differential growth-inhibitors for plants It is claimed that their action is similar to that of colchicine (Arvy and Lhoste, 1946)

The examination of the reaction products of quinones and maleic acid with -SH compounds has been undertaken in order to see whether their action on mitoses would allow us to explain the parallelism between mitotic inhibition and -SH uptake, established experimentally The present investigation affords no clear solution of this problem From the seven adducts examined three have exerted no mitotic inhibition (1, 2, and 3) and four were active as mitotic inhibitors (substances 4, 5, 6, and 7) Dealing with the same problem, Michael (1948) has found unimpaired physiological activity with the quinonoid fuscin after addition of thiolacetic acid On the other hand Kuhn and Bemert (1945), who investigated the inhibition of carboxylase, have shown that Scystemo-p-benzoqu none, resulting from the addition of cysteine to p-benzoquinone, has lost the strong inhibitory activity of p-benzoquinone Furthermore, 2-methyl substitution in 1 4-naphthogumone decreases the antimitotic activity. whereas the introduction of a methyl group in the ortho position in -S-derivatives of 1 4-naphthoquinone increases the antimitotic activity of the new compound as shown in this paper

Simple thiol addition compounds like those we have prepared and investigated may play no part in the mitotic inhibition induced by quinones or maleic acid. No satisfactory evidence is available to discuss other possibilities.

CHEMICAL SECTION

1 S-(I 4-naphthogumonyl-2)-thiolacetic acid

The substance was prepared following the directions given by Fieser and Turner (1947) for the preparation of S-(2-methyl-1 4-naphthoquinonyl-3)-thiolacetic acid 1 4-Naphthoquinone (2 6 g) was dissolved in warm alcohol (120 cc), cooled to room temperature, and mixed with a solution of thiolacetic acid (1 5 g., 1 13 cc, 1 mol quantity) in alcohol (4 cc) After standing overnight the dark solution was evaporated in vacuo when yellow plates appeared

Yield 08 g, mp 173-5° (decomp) after softening at 164° (20 per cent yield of crude product). A portion (05 g.) was dissolved in a solution of sodium bicarbonate (0.5 g.) in water (25 cc) and well extracted with ether. The aqueous phase was acidified to Congo red with 5N sulphuric acid and the precipitated yellow gel was extracted with a large volume (500 cc in all) of ether. The extract was washed twice with water, dried over sodium sulphate, and evaporated to dryness. The crystalline residue was recrystallized from alcohol giving 035 g yellow plates m.p 183.5° (decomp). For analysis, the compound was purified on a column of alumina, when

washing with methanol removed traces of a dark-coloured impurity S-(1 4-naphthoquinonyl-2)-thiolacetic acid was eluted from the column with aqueous bicarbonate, isolated after acidification and recrystallized twice from alcohol to mp 1835 (decomp) Found (in material dried at 80° in vacuo) C, 580, H, 34 $C_{12}H_8O_4S$ requires C, 581, H, 33 per cent

2 S-(1 4-naphthogunonyl-2)-glutathione

The method described by Fieser and Fieser (1944) was used for the preparation of this substance 1 4-Naphthoquinone (316 mg, 1/500 mol) was dissolved in warm alcohol (10 cc) and cooled to room temperature. The solution was added at once to a solution of glutathione (307 mg, 1/1,000 mol) in water (4 cc) and alcohol (6 cc). An immediate darkening occurred with the separation of a yellow solid, which was left overnight at room temperature, filtered off, well washed with alcohol, and dried

Yield 440 mg. yellow amorphous solid (95 per cent yield) Found (in material dried in vacuo at room temperature) C, 516, H, 49, N, 92 C₀H₂₁O₈N₃S

requires C, 518, H, 46, N, 91 per cent

- 3 S-(2-methyl-1 4-naphthoquinonyl-3) thiolacetic acid Prepared by the method of Fieser and Turner (1947), except that after reduction by aqueous hydrosulphite it was found more convenient to re-oxidize to the quinone by means of 10 per cent aqueous ferric chloride containing 1/5 of its volume of 5N sulphuric acid
- 4 S-(2-methyl-1 4-naphthoquinonyl-3)-glutathione Prepared by the method of Fieser and Fieser (1944) in a yield of 86 per cent
- 5, 6, and 7 S-thiolaceto-succime acid (5), S-cysteino-succime acid (6), and S-glutathiono-succime acid (7) have been prepared by Morgan and Friedmann (1938a) The original substances analysed by these authors have been used for our experiments

SUMMARY

- 1 Evidence is given that the mitotic inhibition produced by some quinones and by maleic acid goes parallel with the -SH uptake of these substances
- 2 S-(1 4-naphthoquinonyl-2)-thiolacetic acid (1), S-(1 4-naphthoquinonyl-2)-glutathione

- (2), S-(2-methyl-1 4-naphthoquinonyl-3)-thiolacetic acid (3), S-(2-methyl-1 4-naphthoquinonyl-3)-glutathione (4), S-thiolaceto-succinic acid (5), S-cysteino-succinic acid (6), S-glutathiono-succinic acid (7), have been prepared and investigated in tissue cultures of chick fibroblasts Substances (1), (2), and (3) give no mitotic inhibition, substances (4), (5), (6), and (7) were inhibitors of mitosis
 - 3 The biological results are discussed
- 4 Simple thiol addition compounds to quinones and maleic acid like those mentioned above seem to play no part in the mitotic inhibition induced by some quinones and by maleic acid

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THE ACTION OF CHOLINE DERIVATIVES ON ISOLATED RABBIT AURICLES WHEN ARRESTED BY PALUDRINE

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(Received July 5 1948)

The observation made by Sachs (1937), that minute doses of acetylcholine would stimulate the beat of isolated hearts, has now been repeated by many workers (Spadolini and Domini, 1940, Rothberger and Sachs, 1938, McDowall, 1946, Haney and Lindgren, 1945) Of these Spadolini and Domini were the first to postulate that this action was due to the liberation of an adrenaline-like substance from either ganglia or chromaffin tissue in the heart Hoffmann et al (1945) and McNamara, Krop, and McKay (1948) confirmed that an adrenaline-like substance is liberated from the heart muscle by acetylcholine

In a previous paper from this laboratory (Burn and Vane, 1948), it was shown that if isolated rabbit's auricles were exposed to the action of paludrine, then the inhibitory effect of acetylcholine was gradually changed to a stimulation. This change was accompanied by a gradual reduction in the rate and amplitude of beat. Within 7–38 min after the paludrine had been added the beat of the auricles stopped, usually abruptly, and the auricles remained quiescent even when left in fresh Ringer-Locke solution for periods of up to an hour. During this time acetylcholine restarted the beat. These observations have now been extended to include other acetylcholine-like compounds and adrenaline.

RESULTS

The auricles of a freshly killed rabbit were dissected clean of fat and ventricular tissue, they were then suspended in well-oxygenated Ringer-Locke solution at 29° C, so that the contractions were recorded on smoked paper by a light straw lever, an upstroke representing systole

Acetyl- β -methylcholine —Fig 1a shows the normal inhibitory effect of acetylcholine and acetyl- β -methylcholine on the beat Paludrine (8 mg) was added to the bath of 75 ml Ringer-Locke solution The amplitude and rate of beat steadily declined until they were about 60 per cent of the

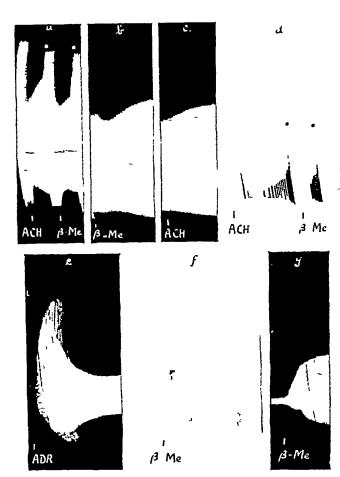


Fig 1—Isolated rabbit auricles (a) Acetylcholine (40 μ g) Acetyl- β -methylcholine (20 μ g) Between (a) and (b) paludrine (8 mg) (b) Acetyl- β -methylcholine (40 μ g) and (c) Acetylcholine (40 μ g) Both caused stimulation The beat stopped (d) Beat restarted with acetylcholine $(200^{\circ} \mu g)^{\circ}$ Stopped when washed out Restarted with acetyl-Stopped when washed β -methylcholine (200 μ g) (e) Restarted with adrenaline (20 μ g) gular beat, which stopped when washed out (f) Restarted with acetyl- β -methylcholine (100 μ g) Continued beating regularly and with larger amplitude than when started with adrenaline (g) Stimulated by acetyl- β -methylcholine (100 μ g) above record indicate that the bath was changed to fresh Ringer-Locke solution

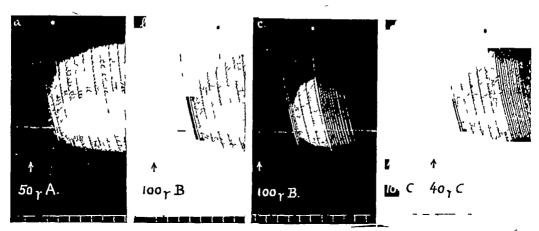


FIG 2—Isolated rabbit auricles stopped by paludrine (8 mg) in 18 min (a) Restarted with acetylcholine (50 μg) and stopped again with paludrine (8 mg) in 4 min (b) Restarted with Bovet's acetal compound (100 μg) Stopped in 4 min with paludrine (8 mg) (c) Restarted with Bovet's acetal compound (100 μg) Stopped when washed out (d) Restarted with carbaminoylcholine (50 μg) Irregular and slow after washing out Time 30 sec Dots above record indicate that the bath was changed to fresh Ringer-Locke solution

initial amplitude (Fig. 1b) At this point acetyl- β methylcholine caused a transient inhibition, followed by a stimulation, of the beat After washing out, acetylcholine also augmented the bear There was no change in rate during these stimulations The bath was replaced by Ringer-Locke solution containing the same concentration of paludrine, the beat of the auricles soon stopped The arrest was not gradual, but Both acetylcholine and acetyl-\(\beta\)-methylcholine restarted the contractions of the auricles (Fig 1d), these stopped when the fluid in the bath was changed to fresh Ringer-Locke solution Acetyl-\(\beta\)-methylcholine was again used to restart the beat (Fig 1f) It was allowed to act for a longer time than before (Fig 1d), and when the bath was washed out the auricles did not stop beating but the beat became gradually much weaker Acetyl- β -methylcholine once more stimulated the beat (Fig 1g) It has been repeatedly noticed in this, and in previous work, that if the substance which initiated the beat was removed within 90 sec of the first contraction, then the beat stopped almost immediately (see Figs 1d and 2c) If the substance was allowed to act for a longer time (2-5 min) then washing out only reduced the beat, and if the drug was left in the bath for more than 5 min washing out had little effect on the beat

Adrenaline —Adrenaline also restarted the beat, as is shown in Fig 1e. The difference between the effect of adrenaline and that of a choline derivative (Fig 1f acetyl- β -methylchol ne) should be noted. Contractions started with adrenaline

quickly reached a large amplitude, but then diminished to a small value. When the contractions were started with an acetylcholine-like compound, after the first few beats the amplitude of the contractions steadily increased to a much larger value than that obtained with adrenaline

Bovet's acetal compound—Bovet (1944) introduced a new compound, which he claimed had the muscarine-like properties, but no nicotine-like properties of acetylcholine The formula of Bovet's acetal compound is shown below

Fig 2 shows that this, as well as acetylcholine, would restart the auricles which had been stopped with paludrine. The auricles were started with acetylcholine (Fig 2a) but did not stop when washed out. Paludrine was added again, and the beat stopped in 4 min. The bath was washed out, and then Bovet's acetal compound restarted the contractions (Fig 2b). Paludrine again stopped the beat in 4 min., Bovet's acetal compound again restarted it. This time the bath was washed out within 90 sec of the initial contraction, and the beat stopped almost immediately.

Carbaminoylcholine—Fig. 2d shows that carbaminoylcholine also restarted the contractions. When the bath was changed, the beat became slow and irregular and did eventually stop

Choline—Choline restarted the beat Fig 3 shows the record of an experiment in which acetylcholine (100 μ g) started the rhythm (Fig 3a) The bath was washed out twice and the contractions stopped in 4 min Choline (5 mg) had no effect, but choline (50 mg) added 90 sec later restarted the beat (Fig 3b) When the bath was washed out, the contractions again stopped, they were restarted with acetylcholine (50 μ g)

Benzoylcholine —Benzoylcholine, which is said to have only nicotine-like properties, failed to restart the contractions of the auricles

Nicotine - Nicotine was added (1, 10, and 50 mg), but this did not restart the beat of the quiescent auricles After the nicotine had been washed out, two doses of acetylcholine (100 µg) were needed to restart the contractions, the force of which was much weaker than before the nicotine The beat stopped when the (Fig 3c) bath was changed, but restarted again with acetylcholine (100 μ g) and became more regular on addition of acetylcholine (200 μ g) Nicotine (0.5, 1, and 2 mg) also failed to restart the contractions of the auricles of which Fig 2 is the record

Atropine —Atrop ne $(500 \mu g)$ added to the bath (Fig 3c) made the beat irregular and the amplitude gradually decreased. The rhythm improved, however, when more acetylcholine was added (1 mg). The effect of atropine on auricles restarted with acetylcholine depended upon the time at which it was added. If the atropine was added within 90 sec of the initial contraction, then the beat stopped immediately. This is recorded in Fig. 4. Acetylcholine restarted the beat, which stopped when the bath was changed. The con-

tractions were again started with acetylcholine, but stopped immediately atropine was added. After the bath had been washed out, although acetylcholine did not restart the beat, adrenaline did, and the contractions were unaffected by atropine

Effect of adrenaline on acetylcholine response— The beat of the auricles was stopped with paludrine and restarted with acetylcholine After 10 min the acetylcholine was washed out, the

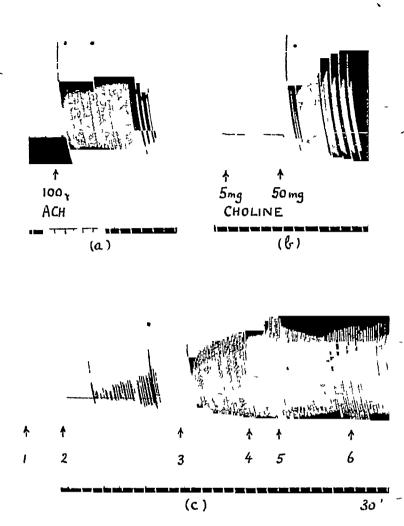


Fig. 3—Auricles stopped with paludrine (8 mg) in 26 min (a) Restarted with acetylcholine (100 μ g), stopped on washing out. (b) Restarted with choline (55 mg), stopped on washing out ϵ Between (b) and (c), restarted with acetylcholine (50 μ g), but failed to restart with nicotine (1, 10, and 50 mg) Washed out (c) Acetylcholine (100 μ g at 1 and 2), started beat, but not so well as in (a) Beat stopped when bath was washed out, restarted with acetylcholine (100 μ g at 3) and improved with acetylcholine (200 μ g at 4) Atropine (500 μ g at 5) made the beat irregular and the amplitude decreased The beat seemed to improve when more acetylcholine was added (1 mg at 6) Time 30 sec Dots above record indicate that bath was changed to fresh Ringer-Locke solution

auricles were left for a further 20 min. The amplitude of the beat was then as shown at the beginning of Fig. 5. Adrenaline increased the amplitude and rate of beat to approximately the value previous to treatment with paludrine, and at this point acetylcholine (10 μ g) inhibited both the amplitude and rate of beat. Without any adrenaline in the bath, acetylcholine (10 μ g and 50 μ g) had no effect on the amplitude, whilst acetylcholine (100 μ g) caused a slight increase. This was accompanied by a decrease in rate. The in-

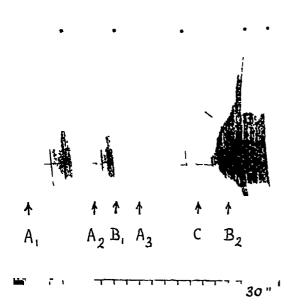


FIG 4—Isolated rabbit auricles The beat was stopped with paludrine (8 mg) in 27 min A_1 Started with acetylcholine (50 μg) Stopped when washed out A_2 Restarted with acetylcholine (50 μg) Stopped on the addition of atropine (500 μg at B_1) A_3 Acetylcholine (50 μg) failed to restart the beat, but adrenaline (20 μg) at C started the contractions, which were unaffected by atropine (500 μg at B_2) Time 30 sec. Dots above record indicate that the bath was changed to fresh Ringer-Locke solution

hibition with acetylcholine (10 μ g) was repeated in the presence of adrenaline

DISCUSSION

The possibility that acetylcholine has an integral function in the mechanism of the heart beat has already been discussed in a previous paper (Burn and Vane, 1948) In this paper it was shown that when the beat of isolated rabbit auricles was stopped with paludrine, it could be restarted with acetylcholine. It is interesting to note that Eurico-Paes and Soares (1940) found that chick-embryo heart-cultures which had ceased to beat could be restarted with a mixture of adrenaline and acetylcholine, but not with either drug separately, and that Singh, Sehra, and Singh (1945) published a record of the resumption of contractions of a frog's heart when acetylcholine was added

It has now been shown that adrenaline and several acetylcholine-like compounds will also restart the beat of auricles stopped with paludrine. That the mechanism of this resumption cannot be due to the release of an adrenaline-like substance (described by Hoffmann et al, 1945, McNamara

et al, 1948) is suggested for the following reasons

- (a) The release of an adrenaline-like substance is a nicotine-like action of acetylcholine. Nicotine did not restart the beat of the auricles, nor did benzoylcholine, which is said to have only nicotine-like properties. Bovet's acetal compound, however, which he claims has no nicotine-like act on, restarted the beat
- (b) When the beat was restarted with adrenaline, in most cases the amplitude was initially stimulated, but then declined and remained small (Fig 1e) Contractions restarted with acetylcholine-like compounds, however, steadily increased to a large value (Fig 1f)

As would be expected, atropine prevented the start of the beat with acetylcholine, but not with adrenaline. If atropine was added to the auricles which had just started to contract under the influence of acetylcholine, then the contractions stopped, if the addition of atropine was delayed until about 5 min after the contractions had started, then it only slowed the beat or had no effect. The same effect could be obtained by washing out, if the bath was washed out within 90 sec of the first contraction, then the beat stopped, if the bath was not washed out until about 5 min. after the first contraction, then the beat continued

Thus the mechanism of contraction seems to be both initiated by, and dependent upon, the added acetylcholine for the first few minutes, the beat then becomes independent of the added acetylcholine. This is interesting in view of the work of Abdon and his collaborators, who found that acetylcholine was present in the form of a precursor in cardiac tissue and suggested that there was a constant breakdown to, and reformation from, acetylcholine. They postulated that this "precursor was necessary for the contractions of the cardiac muscle" (Abdon and Hammarskjöld, 1944, Abdon, 1945)

After treatment with paludrine, the normal inhibitory effect of acetyleholine could be restored by first increasing the force and rate of beat with adrenaline. This suggests that the action of acetyleholine may depend upon the rate of metabolism of the myocardium, or the actions of adrenaline and acetyleholine on the heart are interdependent after treatment with paludrine.

SUMMARY

1 Various drugs have been tested to find whether they will restart the contractions of isolated rabbit auricles, previously stopped with paludrine

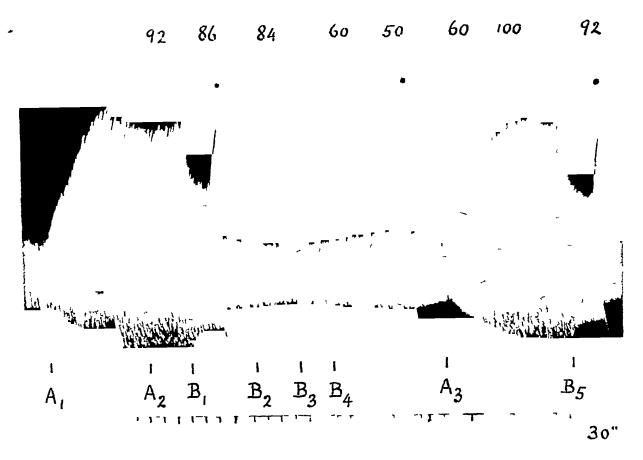


Fig 5—After exposure to paludrine the beat of the auricles stopped, but was restarted with acetylcholine (100 μ g) This was washed out after 10 min. Auricles were left for 20 min. At A₁, adrenaline (20 μ g) increased the rate and amplitude. Further adrenaline (20 μ g at A₂) had no effect, but acetylcholine (10 μ g at B₁) caused inhibition of beat. After washing out, the amplitude returned to its previous value, and at this stage acetylcholine (10 μ g at B₂ and 50 μ g at B₃) gave no inhibition of amplitude, although the rate was decreased. Acetylcholine (100 μ g at B₄) caused slight augmentation of amplitude. After washing out, adrenaline (20 μ g at A₃) again increased the rate and amplitude, acetylcholine (10 μ g at B₅) again inhibited the beat. Figures along top indicate rate of beat per min. Time 30 sec. Dots above record indicate that the bath was changed to fresh Ringer-Locke solution

- 2 Of these drugs, choline, carbaminoylcholine, acetyl- β -methylcholine, Bovet's acetal compound, and adrenaline all restarted the beat
- 3 The beat was not started by nicotine or benzoylcholine
- 4 After treatment with paludrine the normal inhibitory effect of acetylcholine could be restored by first adding adrenaline to the auricles

5 The results are discussed

I am deeply indebted to Professor J H. Burn for his help and guidance throughout this work, which was carried out during the tenure of a grant from the Therapeutic Research Corporation of Great Britain The paludrine was kindly supplied by Dr F L Rose of Imperial Chemical (Pharmaceuticals) Limited

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THE INHIBITORY ACTION OF PALUDRINE ON THE SECRETION OF GASTRIC JUICE

RY

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(Received July 22 1948)

Because excessive secretion of gastric juice is a common concomitant of chronic peptic ulcers, various workers have looked for substances having an inhibitory action. The secretion of HCl. though partly controlled by the vagus nerves. 15 peculiar in being stimulated by histamine and in being not greatly affected by atropine The effect of atropine is described differently by different observers (Polland, 1930, Atkinson and Ivy, 1937, Gray, 1937, King, Comfort, and Osterberg, 1944) It appears to be more effective in abolishing the secretion produced by giving a meal than that produced by injecting histamine Davenport (1940). and later Rehm and Enelow (1945), investigated the effect of sodium thiocyanate, when given intravenously to dogs in sufficient amount, it was found to inhibit the secretion of HCl completely

Recently Babkin and Karp (1947) have observed that the two antimalarial substances quinine and mepacrine (atebrin), when injected intravenously into dogs, depressed the secretion of gastric juice produced by stimulation of the vagus nerves. The doses they used were large in relation to the doses ordinarily given to man, thus a dog of 10 kg received 0.2 g quinine bisulphate or 75 mg, mepacrine. When secretion was produced by the injection of histamine, neither quinine nor mepacrine affected it

Recently a simple method of examining substances for an inhibitory action on gastric secretion has been developed in this laboratory by Wood (1948), cats are used, anaesthetized by cyclopropane or pentobarbitone (nembutal) The only essential point in which the method differs from that proposed by Lim (1923), and modified by Roth and Ivy (1944), is that histamine solution has been infused into the jugular vein at a uniform rate for periods up to 7 hours. In order to obtain a uniform infusion, a pump designed and made by Dr E H J Schuster has been used, with which a reasonably steady secretion of gastric juice has

been obtained The oesophagus was tied in the neck and not at the cardiac orifice, to avoid the vagi

The action of paludrine

During the examination of the pharmacological properties of paludrine, its effect on the secretion of gastric juice was tested by this method. When histamine was infused, the rate of secretion rose gradually until at the end of about 1½ hours the amount collected in each 10 min period became fairly steady. Sometimes 4 hours passed before the flow was steady. At this point, while the histamine infusion continued, the effect of paludrine

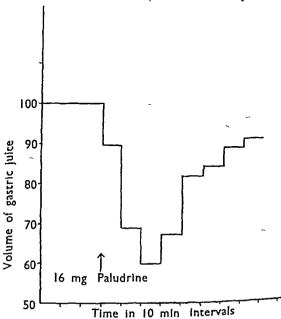


FIG 1—To show the fall in the secretion of gastric juice when 16 mg paludrine were injected. The mean effect on the volume of juice in 9 cats is shown. In each experiment the initial secretion has been given the value 100. Abscissae time in 10 min periods.

was examined by making an injection of a single dose intravenously, or else by infusing paludrine intravenously It was found that paludrine in doses which had only a transient effect on the blood pressure diminished the volume of juice secreted for a period of 60-90 min The results of nine experiments in which a single injection of 16 mg paludrine was given were combined so that the mean effect could be determined is shown graphically in Fig. 1, the volume of juice excreted per 10 min during 30 min, before paludrine was injected is expressed as 100, and the volume fell to 60, after the injection After reaching a minimum the flow gradually returned to its previous value, and the effect of paludrine could then be observed a second time Details of one experiment were as follows Histamine was in-

fused at the rate of 15 μ g per min. into a cat of 3 2 kg under nembutal anaesthesia (25 mg per kg) The rate of flow of gastric juice was 26 ml per 10 min during 30 min, and after the injection of 16 mg paludrine it fell to 16 ml during 65 min The rate then returned to 26 ml for a further 60 min. In four experiments the injection of 8 mg paludrine caused a smaller mean reduction in the flow This effect to 75 per cent of the initial value When larger amounts of paludrine were given

by slow infusion instead of by single injection, the periods of reduced gastric secretion lasted until the termination of the experiment Fig 2 shows the mean effect of infusing 60 mg paludrine during 30 min into 6 cats The graph records the change in the total free acid excreted The volume of juice per 10 min. was measured, 1 ml of this juice was then titrated against N/50 NaOH using thymol blue as the indicator The total secretion of free acrd per 10 min was then calculated initial rise in secretion shown in Fig 2 occurred in two of the six experiments for 20 min, thereafter there was a prolonged fall In these experiments, both the volume of juice secreted and the concentration of free acid in the juice were re-The only effect the paludrine infusion had on the blood pressure was to cause an occasional In four other experiments, doses of paludrine from 120 mg to 180 mg were infused Table I shows these results It can be seen that there was an initial stimulation of the secretion in In these experiments, as in those recorded in Fig 2, the volume of juice secreted did not recover from its low value

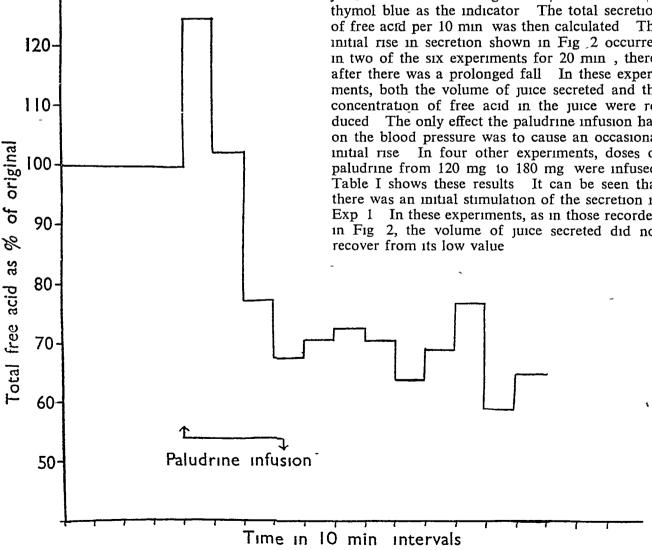


Fig 2.—To show the mean fall in total free acid in gastric juice caused by intravenous infusion of 60 mg paludrine into 6 cats. In 2 experiments there was an initial increase in secretion.

	TABLE I		
EFFECT OF INFUSED	PALUDRINE ON VOLUME O	OF GASTRIC	JUICE IN CATS

Exp	Rate of hista- mine infusion µg/min	Paludrine mg	Duration of infusion min	Gastric juice per 10 min ml.	Period of observation min	Mean percentage to which volume of juice fell
1	15	140 "	52	63 87 43	60 20 110	138 68
2	15	120	100	4 6 3 8	30 140	82
3	15	180	90	4 7 2 2	60 160	- 47
4	15	120	60	1 7 0 5	40 170'	29

In all experiments the rate of histamine infusion was usually 15 μg per min, or more, throughout, Only in two experiments was it 10 μg per min. This is a high rate of histamine infusion, providing a powerful stimulus to secretion and a severe test for an inhibitory substance

Paludrine consists of the biguanide molecule, substituted at one end with the p-chlorophenyl radical, and at the other with the isopropyl radical Compounds structurally related to paludrine were also tested to see if they would inhibit gastric secretion. The first three compounds tested were biguanide, isopropyl biguanide, and di-isopropyl biguanide. All were found to be inactive as shown by the results in Table II. These results were obtained by expressing the total secretion for an hour after the drug was given as a percentage of

TABLE II
NON-INHIBITORY BIGUANIDES

Substance	Dose in mg.	Total free acid for 1 hr after drug as % of that for 1 hr before drug	Average
Biguanide	60 60 60	133 91 84	106
Isopropyl- biguanide	60 60 60 60	140 106 103 57	102
	120	- 89	
Di-isopropyl- biguanide	60 60	120 93	107

the total secretion of the hour previous to the administration

In an effort to obtain a more quantitative estimation of the inhibitory power, the principle of the method recently described by Howat and Schofield (1948) was adopted. The main difference between this method and that previously used was that, instead of giving the test substance during a constant infusion of histamine, it was given between two periods of histamine infusion, each of 48 min duration. The second response was expressed as a percentage of the first, the

TABLE III
PALUDRINE AND RELATED COMPOUNDS

Substance	Dose in- fused mg	Total free acid expressed as % of original value	Aver-
Paludrine (N ₁ -p-chlorophenyl- N ₅ -isopropyl biguanide)	60 60 60	75 71 17	5 4
N ₁ -p-chlorophenyl- N ₆ -methyl-biguanide	60 60 32	66 \ 32 \ 68	49
N ₁ -p-chlorophenyl- biguanide	80 60 60	92 33 155	93
N ₁ -p-methoxyphenyl- biguanide	60 60	78 64	71
N ₁ -p-chlorophenyl- N ₃ -methyl-guanidine	60 60 60	141 70 23	78

TABL	E	IV
SUMMARY	OF	RESULTS

NH—C	Amount (mg) administered	No of expts	Approximate percentage to which secretion was reduced	
Cl	—C ₃ H ₇	60	9	60
Cl —	—CH ₃	60	2	50
CH ₂ O —	—Н	60	2	70 -
Cl —	—Н *	. 60	3	90
C₃H ₇ — C₃H ₇ — H—	—H —C ₃ H ₇ —H	60 60 60	4 2 3	no reduction
CI —NH-	-С—NН—СН, NH	60	3	80

figure being a measure of the inhibitory power of the test substance

By this method, five more compounds were tested (including paludrine as a standard) The results of these experiments are shown in Table III The mean secretion of acid in three experiments in which paludrine was infused was reduced to 54 per cent of the original value, by the first method, six experiments gave a mean value of 64 per cent. The results shown in Tables II and III have been combined and arranged in order of activity, to give Table IV

DISCUSSION AND SUMMARY

Extensive trials of paludrine in men have been made in testing its value against malaria, and it is known to be a well-tolerated substance A large dose produces gastro-intestinal symptoms in some individuals, and Hughes, Schmidt, and Smith (1947) have described how dogs fed on a diet containing paludrine become disinclined to eat. It seems probable that these observations are related to our finding that paludrine exerts an inhibitory action on the volume and acidity of gastric juice evoked by histamine A similar compound in which the isopropyl group of paludrine was replaced by a methyl group was found to exert a similar effect Other related substances had less or no effect We have observed an initial stimulating action in a few experiments, preceding the inhibition. This is probably an example of a preliminary stimulant action preceding inhibition, which is commonly met in inhibitory drugs. Thus the first effect of taking atropine by mouth is to slow the pulse rate and cause the hands to become moist in most people, the later effect is of course the opposite. Atropine appears first to stimulate and later to paralyse the cholinergic nerve endings in the heart and sweat glands.

We are greatly indebted to Dr F L Rose, of Imperial Chemical (Pharmaceuticals) Limited, for the substances which we have examined The work was done while one of us (J R V) was in receipt of a grant from the Therapeutic Research Corporation

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THE EFFECT OF PALUDRINE ON GASTRIC SECRETION IN MAN

BY

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In order to investigate the effect of paludrine on human gastric secretion the following experiments were carried out on 9 students. Each subject, who had not taken food for at least four hours, had his stomach emptied by aspiration. He was then given a meal of 450 ml gruel, and samples of gastric contents were withdrawn every 15 minutes for 2 hours. The hydrochloric acid content of each sample was measured by titration against standard sodium hydroxide using thymol blue as indicator. Three control experiments were first carried out on each subject in order to obtain his normal response.

Paludrine was given by mouth, either as tablets or in cachets, 2 or 4 hours before the test meal. The only toxic effect was slight nausea in four subjects after the higher doses, and this passed off before the meal was given. Results were assessed as follows (see Table I). The amount of

TABLE I

STUDENT H

The figures in columns (ii)—(vii) are ml N/10 HCl

per 100 ml gastric juice

(i)	(ıi)	(111)	(iv)	(v)	(vi)	(vii)
Controls			Mean	Paludrine		
Mın	1	2	3	of con- trols	0 9 g. 4 hr before	10 g 2 hr before
15 30 45 60 75 90 105 120	0 23 43 67 80 73 69 43	0 15 58 80 78 73 32	8 40 40 80 50 58 85 57	2.8 26 0 47 0 76 0 69 3 68.2 62.0 50 0	8 28 50 83 100 80 53 65	8 22 48 65 53 42 25 37
Mean	49 7	48 0	52 2	50 1	58 4	37 5

HCl in each sample of gastric juice, expressed as ml N/10 HCl per 100 ml juice, is set out in each column, and the mean of these figures is shown at the bottom of the column Mean figures of the three control experiments are given in column (v) The results after paludrine are given in columns (vi) and (vii) and can be compared with those of the controls

Table II shows the results obtained on each student in this way. It can be seen that when paludrine was given 4 hours beforehand (columns (vi) and (vii)) there was usually no effect. In one case, student J, the acidity was markedly increased When, however, 09-10 g paludrine was given 2 hours before the test meal (columns (viii) and (ix)) there was a decrease in the gastric acidity of every subject but one. The mean figure for the acidity of the samples when this dose of paludrine was given two hours before the test meal was 230 ml per 100 ml juice. This is 60 per cent of the mean figure of the control samples (381 ml)

TABLE II

EFFECT OF PALUDRINE ON GASTRIC SECRETION OF HCl

The figures are ml N/10 HCl per 100 ml gastric juice

(1)	(11)	(m)	(1V)	(v)	(v1)	(11)	(vni	(17)
Controls		Mean of	Dose of paludrine Time before meal					
ject	1	2	3	con trols	0 3 g 4 hr	09g 4hr	0 9g 2 hr	1 0g 2 hr
A B C D F G H J	20 45 28 52 46 18 28 49 25	35 45 39 33 71 41 49 48 26	10 30 26 33 73 46 17 52	22 41 32 39 64 37 33 50 25	28 37 43 37 36	26 45 29 82 13 58	13 14 37	8 26 27 25 47 17 11 28
Mean	34 5	43 0	34 5	38 1	38 6	43 8		23 0

An analysis of variance of the figures in Table II shows that this reduction of acidity was highly significant (P=<0.01) by the variance-ratio test.

SUMMARY

1 The effect of paludrine on gastric acidity has been investigated by carrying out test meals on 9 subjects

2 A significant reduction in acidity occurred the Medical Research Council

when 09-10 g paludrine was given by mouth 2 hours before the test meal

We are grateful to the nine students who volunteered to act as subjects in these experiments

This work was done when one of us (J R V) was receiving a grant from the Therapeutic Research Corporation of Great Britain, and when another of us (J M W) was receiving a personal grant from the Medical Research Council

THE EFFECT OF PALUDRINE ON HUMAN GASTRIC SECRETION

BY

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Burn and Vane (1948) have reported that paludrine depressed gastric secretion in cats after histamine stimulation. This was worth investigating in man, as paludrine is comparatively nontoxic and a means of reducing gastric secretion might be of value in the treatment of peptic ulcer

Doses of up to 15 g a day have been given without producing serious toxic effects. Abdominal discomfort, nausea, and vomiting have been noted by Fairley (1946) and Maegraith (1946) with doses of 10 g a day, and Fairley also observed diarrhoea and haematuria. The toxic symptoms usually subsided without reduction of the dose as the malaria for which the paludrine was given was relieved.

We have studied the effect of paludrine on gastric secretion in man by two methods, giving it orally before a gruel test meal and intravenously with subcutaneous injections of histamine

Twenty patients (11 men and 9 women) were given gruel test meals on two consecutive days, on one of which they received 10 g paludrine hydrochloride by mouth 2 hours before the meal began. One man and one woman were excluded from the series on account of achlorhydria in both test meals, and the tests could not be completed in two women who vomited after the paludrine. The results in 16 cases are available for analysis

Paludrine was given before the first meal in half the cases and before the second in the other half. The resting juice was drawn off immediately before the meal was started, and samples were taken at half-hourly intervals up to two and a half hours. The concentration of free acid after paludrine was compared with that of the sample drawn off after the same interval in the control meal. The averaged results for the 16 cases are shown in Table I

TABLE I
The effect of 10 g paludrine hydrochloride on the concentration of free HCl after a gruel test meal Mean results for 16 patients

Time		HCl per astric juice	B—A	دري،	
	A B Paludrine		D-A		
½ hr 1 hr 1½ hrs 2 hrs 2½ hrs	18 1 30 8 37 7 30 6 22 3	77 173 254 255 237	-10 4 -13 5 -12.3 -5 1 +1 4	2 01 2 17 2 31 1 12	
Average	_ 27 9	19 9	-≉0	3 78	

Individual results varied from an increased concentration of acid after paludrine to the production, in four cases, of achlorhydria Considering them as a whole, the average depression of free acidity after paludrine was 80 cc N/10 HCl per 100 c c juice Applying the "t" test to the 80 individual differences between comparable specimens, the probability of obtaining such differences due to chance on the hypothesis that paludrine had no effect is less than 0.01 (t=3.78, n=79) depression can therefore be considered to be real If the results at each period after the start of the meal are considered separately a significant depression with paludrine was found at 1 and 1½ hours (0 02 < P < 0 05), while after a half-hour the depression was just too small to be considered No effect was demonstrated at 2 and significant 2+ hours

Repetition of the test meal had no effect on the acidity of the gastric secretion. The average acidity of all the samples drawn off during the second meal was 0 3 cc. N/10 HCl per 100 cc.

^{*} While working with a grant from the Medical Research Council

less than the average of the samples of the first meal

In contrast to the results with oral paludrine and gruel test meals, no effect was demonstrated when paludrine was given intravenously and gastric secretion was stimulated with histamine subcutaneously, 0.5 mg histamine was given initially, with a further 0.5 mg after 60 to 80 minutes. Doses of up to 400 mg paludrine acetate were given intravenously immediately before the second injection of histamine. The stomach was emptied at 10 min. intervals, and the effect of the paludrine was determined by

TABLE II

The amount and concentration of free acid secreted in one hour after 0.5 mg histamine (1st hr.) and 0.5 mg histamine S.C. and 400 mg paludrine I.V (2nd hr.)

Sub-	Amount of free acid, cc N/10 HCl			of free	ge concer acid, c o	: N/10
jui	1st hr	2nd hr	Differ- ence	1st hr	2nd hr	Differ- ence
1 2 3 4	202 1 203 0 14 1 254 7	155 4 181 9 19 9 323 3	-467 -211 +58 +686	59 0 67 9 20 0 107 0	59 1 65 7 - 37 0 113 2	+01 -22 +170 +62

comparing the amounts and average concentrations of free acid secreted in the hours following the first and second injections of histamine. The results in the 4 subjects given 400 mg are shown in Table II

The amount of paludrine given intravenously was small in comparison with that used by Burn and Vane in cats, but it was comparable with the oral dose which was effective in depressing acid secretion in response to a gruel test meal in man The reason for the conflicting results is not clear

SUMMARY

10 g paludrine hydrochloride given 2 hours before the start of a gruel test meal produced a significant depression of more than 33 per cent in the concentration of free acid during the first 1½ hours of the meal No consistent effect was observed with doses of up to 400 mg paludrine acetate given intravenously on the gastric secretion in response to histamine

We should like to express our thanks to Dr F Avery Jones, under whose direction the work was carried out, for his help and advice

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THE ACTION OF ANTAGONISTS OF ACETYLCHOLINE ON THE VESSELS OF THE RABBIT'S EAR

BY

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Our interest in the work to be described began with an observation made in this laboratory by H W Ling, that when the vessels of a dog's hind leg were perfused with defibrinated blood containing adrenaline, the injection into the cannula of atropine sulphate caused vasodilatation and increased blood flow through the leg. The observation led to an investigation of the vascular action of atropine by Bussell (1940), who found in the course of his observations that the constrictor action of adrenaline on the vessels of the rabbit's ear, perfused with Locke's solution, was abolished by atropine, though atropine did not affect the constrictor action of posterior lobe extract

Since that work was done, fresh light has been thrown on the action of atropine by the work of Dawes (1946), who showed that it exerted a quinidine-like action on the rabbit auricles, and that its properties were shared in varying degrees by other substances, including local anaesthetics such as procaine, and the analgesic pethidine These substances revealed themselves as antagonists of acetylcholine, and a comparison of the properties of some of them was made by de Elío (1948), a study of their effect on body temperature was also made by Dutta (1948) It remained to investigate their vascular action and to observe in particular whether like atropine they would abolish the constrictor action of adrenaline in the vessels of the rabbit's ear The substances used have been procaine, pethidine (demerol), benadryl, quinidine, and atropine, though a few observations have also been made with antistin, anthisan (neoan-Since Gowdey (1948) tergan) and isoconessine had observed that priscol (benzylimidazoline) reversed the action of adrenaline in the rabbit's ear, we studied the effect of this substance also

METHODS

All observations were made on the vessels of the rabbit's ear perfused with Locke's solution. The method of Gaddum and Kwiatkowski (1938) was used first in order to stimulate the postganglionic sympathetic fibres. Later experiments were carried out by perfusing the ear severed from the head according to the method of Rischbieter (1913) (originally introduced by Bissemski). The outflow recorder described by Stephenson (1948) was found well suited to record small changes in the diameter of the vascular bed, which would not have been observed using the drop-timer.

RESULTS

The abolition of the vasoconstrictor action of adrenaline

Injections of atropine sulphate, benadryl, pethidine hydrochloride (demerol), procaine hydrochloride, and quinidine hydrochloride were found to diminish or abolish not only the vasoconstriction produced by the subsequent injection of adrenaline but also that produced by sympathetic nerve stimu-These effects are illustrated in Figs 1 lation In Fig 1 the effect of 200 µg pethidine was transitory, being present 2 min after its injection but absent 8 min later In Fig 2 the effect of 500 μ g quinidine was still present, though fading, 28 min after its injection (1940) had previously observed that procaine antagonizes adrenaline and sympathetic nerve stimulation on blood vessels The amounts of the different substances required to reduce the effect of sympathetic nerve stimulation were in general the same as those required to reduce the effect of adrenaline, but this was not true for atropine Whereas 5 µg atropine abolished the effect of

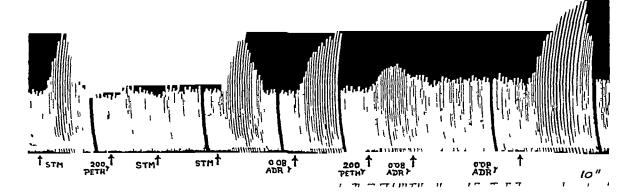


Fig 1—Rabbit ear perfusion using Gaddum's drop-timer Vasoconstriction produced by sympathetic stimulation, after injection of 200 µg pethidine, one stimulation was without effect, the next stimulation was again effective Vasoconstriction produced by 0.08 µg adrenaline, 200 µg pethidine when injected caused some vasoconstriction, the next injection of 0.08 µg adrenaline was without effect, but the succeeding injection produced augmented vasoconstriction

TABLE I

Adrenaline hydro- chloride	Histamine acid phosphate	Vasoconstriction abolished by
0 015 μg	0.2	5 μg pethidine hydrochloride
01 μg	0.2 μg	10 μg ,, ,, 0 5mg procaine hydrochloride
0 01 μg	0 5 μg	0.2 mg ,, ,, $4 \mu \text{g}$ benadryl
	0 01 μg	$0.02 \mu g$,,
0 075 μg	0 2 μg	30 μg quinidine hydrochloride 0 3 mg , , ,
$0015\mu\mathrm{g}$		$4 \mu g$ atropine sulphate
	0 2 μg	6 μg ", ",

To ois y STM SOON STM STM STM ADR. STM

Fig 2—Record as Fig 1 Vasoconstriction produced by 0 013 μ g adrenaline and also sympathetic stimulation Injection of 500 μ g quinidine caused vasoconstriction, and then stimulation was twice ineffective Later 0 013 μ g adrenaline caused much less vasoconstriction than before, and stimulation a similar reduced effect.

adrenaline, 2 mg was required to abolish that of sympathetic nerve stimulation

Observations were also made on the vasoconstriction produced by histamine, this was also abolished by these substances The figures given in Table I illustrate the order of the amounts required to abolish the effects of adrenaline and Against adrenaline, histamine benadryl, and pethidine were strongest, quinidine next, and procaine weakest Against histamine, benadryl was strongest, then atropine and pethidine, and finally procaine and quinidine weakest Anthisan (neoantergan) and antistin were also observed to abolish the constrictor action of adrenaline

The abolition of the vasodilator action of adrenaline

The reversal of the constructor action of adrenaline to a dilator action by perfusing priscol in a concentration of 02 mg/ml (Gowdey, 1948) made it possible for us to examine the effect of these compounds on the dilator action of adren-For these experiments we used Stephenson's recorder We observed that atropine, benadryl, and pethidine abolished the vasodilator action, the effect of pethidine is illustrated in Fig. 3 We were not able to observe a similar action of procaine and of quinidine because when injected in the presence of priscol they themselves caused vasodilatation, which made it difficult to determine the effect of adrenaline When, however, a succession of small doses of quinidine was injected, it was possible to see

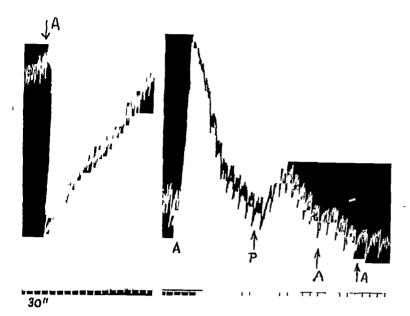


Fig. 3—Outflow record from ear using Stephenson's recorder Vasoconstriction due to 0.004 μg . adrenaline (A) Between t e two parts of the record priscol was added to perfusion fluid (200 μg /ml), and adrenaline (0.008 μg) at A then caused dilatation At P, 10 μg . pethidine hydrochloride was injected, and 0.008 μg adrenaline was then twice injected at A, it had no effect.

that the vasodilator action of adrenaline was reduced

Substances causing adrenaline reversal

We found evidence in the literature that some of the compounds we were considering reverse the constrictor action of adrenaline Thus Wehland (1924) found that, in the perfused vessels of the frog, atropine reversed the action of adrenaline Further the observation of Murakami (1930) and Akamatsu (1933) that quinidine reversed the action of adrenaline on the blood pressure of the cat was supported by the evidence of Keogh and Shaw (1943), who found that quinine reversed the action of adrenaline but that reversal by quinid ne was more difficult to produce therefore carried out experiments on the ear vessels perfused by Locke's solution containing quin.dine hydrochloride (20 μ g per ml) and found that the constrictor action of adrenaline was reversed, as shown in Fig 4 A similar reversal was also observed when the perfusion fluid contained benadryl (10 µg per ml) or procaine hydrochloride (200 μ g per ml) The effects of pethidine hydrochloride and of atropine sulphate were less clear, for when perfused in a concentration of 10 μ g per ml and 30 μ g per ml respectively,

the constrictor phase was not wholly abolished, and the vasodilatation which followed was not so well marked as with the other substances. The vasodilatation was nevertheless present, as shown in Fig 5. In one experiment we confirmed Rothlin's observation (1925), that ergotamine reversed the action of adrenaline, since we observed a reversal with ergotoxine.

Vascular action of benadryl, pethidine, etc /

In the course of these experiments we observed that the injection of benadryl or of the other substances had an effect on the vessels In Fig 1 the second injection of pethidine had a small vasoconstrictor effect and in Fig 2 the injection of quinidine caused vasoconstriction All except atropine were found to cause constriction, and we observed in addition that the constriction reversed to dilatation when priscol was present in the perfusing fluid priscol, During perfusion with

atropine also caused dilatation. These effects are illustrated in Figs 6 and 7. (In one experiment in a freshly prepared ear, benadryl caused slight vasodilatation in the absence of priscol. It seems to us probable that a dilator effect can be observed with these substances at an early stage, but that

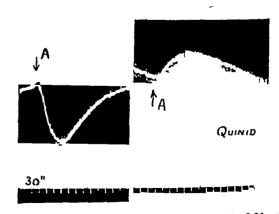


Fig 4—In the first part of the record, at A, 0.01 µg, adrenaline caused vasoconstriction. Between the two parts of the record, quinidine hydrochloride was added to the perfusion fluid (20 µg,/ml) and in the second part 0.05 µg adrenaline at A caused vasodilatation.

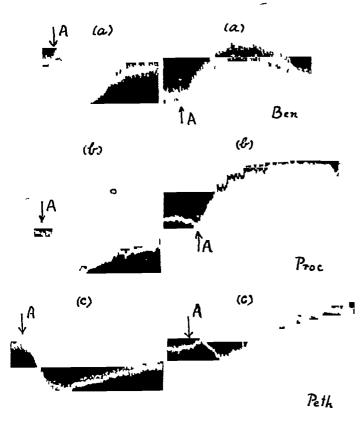


Fig 5—Similar to Fig 4 Reversal of adrenaline (A) Action due to (a) benadryl perfused in concentration 10 μ g/ml, (b) procaine 200 μ g/ml, and (c) pethidine 10 μ g/ml In (a) 0 005 μ g adrenaline was injected before and also during benadryl infusion In (b) 0 01 μ g adrenaline before procaine, 0 02 μ g adrenaline during procaine infusion In (c) 0 005 μ g adrenaline before pethidine, 0 01 μ g adrenaline during pethidine infusion

this gives place as perfusion continues to a constrictor effect, as with acetylcholine This requires further investigation)

Action of acetylcholine and histamine

30"

When the vessels of the rabbit ear are freshly prepared it is usual to observe that acetylcholine causes vasodilatation during the first 8 hours. At the end of 24 hours, this dilator response usually changes to a constrictor response. We observed that this constrictor action of acetylcholine was also reversed to a dilator action when the perfusing Locke's solution contained priscol (0.2 mg/ml). This is shown in Fig. 8. On the other hand the constrictor action of histamine was not reversed, though it was reduced by priscol, thus, before adding priscol to Locke's solution for the perfusion, constriction was produced by 0.03 µg

histamine acid phosphate, during priscol perfusion constriction was produced by amounts from $0.25-1.0~\mu g$

Exceptional actions of adrenaline and histamine

When adrenaline and histamine are injected constriction is the ordinary response in the freshly prepared organ. We have, however, observed a dilator response to adrenaline and to histamine In one rabbit ear the vessels were exceptionally in high tone when the perfusion began, and it was necessary to raise the Marriotte bottle to a height of 75 cm Gradually the perfusion became faster, but the resistance remained considerable and for several hours dilator responses to adrenaline and histamine were consistently recorded, as well as to acetylcholine These responses are illustrated in Repeated control observations were made with Locke's solution On the next morning, however, the dilator responses had disappeared, and all three substances caused vasoconstriction

In another ear, when freshly prepared, it was observed that adrenaline caused constriction followed by dilatation of rather greater extent than the constriction, and did so after several injections. Here again histamine also caused dilatation, though the effect was very small

DISCUSSION

Adrenaline is commonly described as a vasoconstrictor substance, histamine and acetylcholine as vasodilator substances Yet so long ago as 1918 Dale and Richards showed that, when injected into the vein of a cat anaesthetized with ether, all three substances caused the expansion of the volume of a fully denervated hind leg, the expansion being accompanied by a fall in blood pressure Evidently, under the conditions described, all three substances acted as vasodilators If, on the other hand, these substances are injected into the Locke's solution perfusing the vessels of the rabbit ear, the perfusion having continued for 24 hours, then each substance causes vasoconstriction. It is therefore clear that all three substances can act either as vasoconstrictors or as vasodilators, according to the conditions in which they are applied

Some evidence suggests that the vasodilator effect of adrenaline, which has been observed only in vivo, may not be due to adrenaline itself but to the release of histamine by adrenaline. Thus Staub (1946) has shown that the injection of adrenaline causes a rise in the amount of h stamine in the blood. It seems now reasonably certain that adrenaline has a direct vasodilator action of its own. The observation of Rothlin (1925), that in

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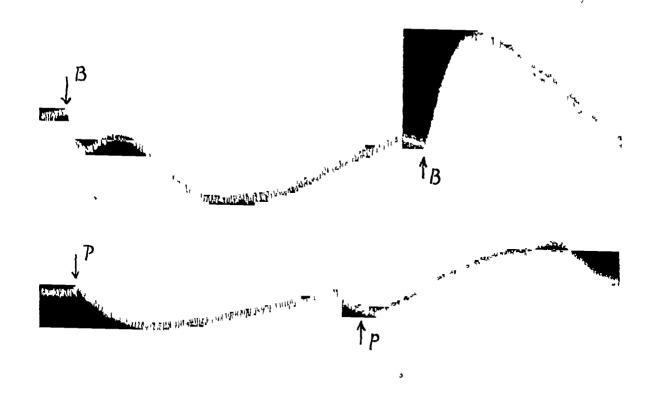


Fig 6—Upper tracings show action of benadryl (B) 100 μ g benadryl produced constriction, during perfusion with priscol (200 μ g /ml), benadryl (10 μ g) produced dilatation. Lower tracings show action of pethidine (P) 2 μ g pethidine caused vasoconstriction, during perfusion with priscol, pethidine (10 μ g) produced dilatation

the presence of ergotamine adrenaline dilates the vessels of the perfused rabbit ear, has been confirmed by us using ergotoxine, and our colleague Gowdey (1948) has observed that in the presence of priscol adrenaline dilates the vessels of the rabbit ear During priscol infusion the constrictor action of histamine itself was reduced but never reversed, so that the dilatation caused by adrenaline could not be due to histamine important, however, is our observation that in two fresh preparations adrenaline produced vasodilatation without the injection of any reversing agent In one of these the vessels responded by dilatation during 8 hours observation not only to adrenaline but to histamine and acetylcholine as well other substance than these three was injected at any time, the perfusion with Locke's solution was continued during the night, and on the following morning the response to the injection of adrenaline, histamine, and acetylcholine had become

constrictor In the second preparation the injection of adrenaline caused an initial constriction followed by dilatation, this was also observed several times. In the course of 20 preparations a vasodilator action of acetylcholine has almost always been observed during the early stages of perfusion, but repeated vasodilator responses to adrenaline and histamine have been seen in two preparations only

There is, as Bülbring and Burn (1948) have found, a steady change in the response of the perfused vessels of the rabbit ear to the injection of acetylcholine, the response, which is at first dilator, becomes constrictor. There may be a similar change in the response to adrenaline, for although the initial response is almost always constrictor the vessels become much more sensitive with continued perfusion so that at a later stage a given dose produces more constriction than before. We have observed that this increased

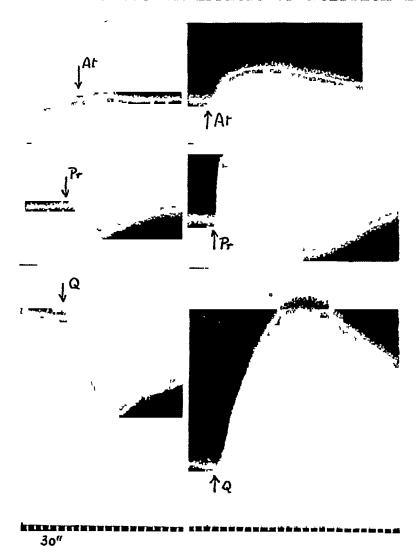


Fig 7—Similar to Fig 6 Upper tracings 50 μg atropine (At) before, and 100 μg atropine during, priscol infusion Middle tracings 500 μg procaine (Pr) before and also during priscol infusion Lower tracings 200 μg quinidine (Q) before, and 100 μg during, priscol infusion

sensitiveness is reduced by perfusing a steady concentration of acetylcholine through the vessels, so that we do not think that the increased sensitiveness is due to oedema

The view that the dilator effect of adrenaline or histamine is exerted on a different part of the vessels from that where these substances produce vasoconstriction has long been held, such a view is less easy to hold when the system of vessels is as simple as that of the perfused rabbit ear. It seems at least equally probable that both effects are produced at the same site. Adrenaline stimulates the smooth muscle of the isolated rabbit uterus, but in the presence of ergotoxine adrenaline causes relaxation, in this tissue it is very unlikely

that there are two sites of action and much more likely that contraction and inhibition are produced at the same recep-Both stimulation and inhibition by adrenaline were observed by McSwiney and Brown (1926) in several other tissues in which the site of action for both effects was probably the same Recently Burn and Vane (1948) have demonstrated that acetylcholine causes both stimulation and inhibition in the isolated rabbit auricles after treatment with paludrine, and also in the isolated rabbit intestine and the isolated rat uterus These opposed effects presumably occur at the same site in each organ, and we therefore favour the view that the two effects of adrenaline in blood vessels are also produced at the same site

The evidence is stronger that the dilator and constrictor effects of histamine are produced at different sites, though Burn and Dale (1926) observed that histamine caused dilatation of the perfused mesenteric artery of the dog with its fine arterial branches, thus showing that dilatation is not produced by histamine in capillaries only

Our observations also indicate the close relation which exists between the action of acetylcholine and that of adrenaline on the blood vessels. Substances such as atropine, pethidine, quinidine, benadryl, and procaine, which inhibit the constrictor action of acetylcholine, also inhibit the constrictor action of adrenaline in the rabbit ear. The close relation is also shown by the observation that the perfusion of priscol through the vessels reversed not only the constrictor action

of adrenaline but that of acetylcholine as well Vasodilatation thus produced by adrenaline was reduced or abolished by a preceding injection of

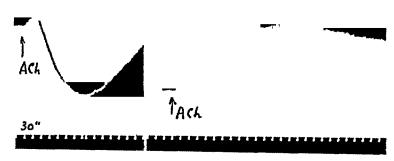


Fig. 8—Reversal of constrictor action of acetylcholine by priscol 10 μg, acetylcholine before, and 100 μg, acetylcholine during, priscol infusion

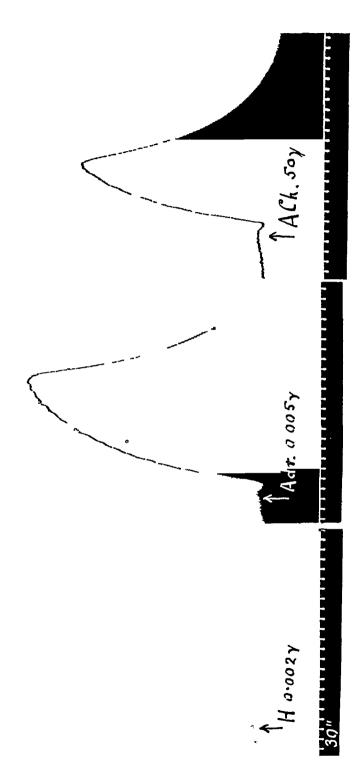


Fig. 9—Exceptional dilator effects of histamine acid phosphate, $0.002 \mu g$ (H), and of adrenaline hydrochloride, $0.005 \mu g$ (Adr), in freshly prepared ear. The usual dilator effect of acetylcholine chloride, $50 \mu g$ (Ach), is also shown. After 24 hours all three substances caused vasoconstriction in this preparation

substances like benadryl, pethidine, and atropine - action of adrenaline and sympathetic stimulation

Möller (1937) has observed that procaine constricts the vessels of the rabbit ear, and we found that large doses of benadryl, pethidine, and quinidine also cause vasoconstriction, these constrictor effects are reversed like that of adrenaline by per-It was surprising fusing the vessels with priscol to observe that quinidine reversed the vascular action of adrenaline like ergotoxine formed the experiment because Keogh and Shaw (1943) described an adrenaline reversal in the cat under nembutal anaesthesia following the injection Having observed an adrenaline reof quinine versal by quinidine in the vessels of the rabbit ear, we proceeded to test the other substances in the same way, and found that when these are perfused in constant concentration through the vessels, the constrictor action of adrenaline is reversed by benadryl, procaine, and even pethidine, though the initial phase of the constrictor action is not completely abolished by pethidine substances which reverse the action of adrenaline have been hitherto regarded as a group apart, but our findings indicate that the properties first described for ergotoxine are properties shared by substances as dissimilar as quinidine and benadryl

These additional observations that benadryl, procaine, pethidine, and quinidine are themselves constrictor substances which, like adrenaline or acetylcholine, are reversed in their vascular action by priscol suggests that when they inhibit the action of adrenaline or acetylcholine they do so because of their adrenaline-like or acetylcholine-like property, that is to say, the inhibition is due to competit on for the same receptors

If it is true that they compete for the same receptors, how is their ergotoxine-like property to be explained? The constrictor action of adrenaline is, of course, a motor effect, and the dilator action after ergotoxine is an inhibitor effect and Vane (1948) have suggested that with acetylcholine the transition from a motor to an inhibitor effect always occurs when excess of it is present Such an explanation may apply to adrenaline the addition of benadryl or pethidine simulates the addition of a maximal amount of adrenaline, then the further addition of adrenaline would cause inhibition

SUMMARY

Earlier work has shown that a group of substances which includes atropine, benadryl, pethidine (demerol), procaine, and quinidine have the common property of antagonizing the action of Experiments on the perfused acetylcholine vessels of the rabbit's ear have now shown that these substances will also inhibit the constrictor

- When the constrictor action of adrenaline is reversed by priscol, these substances, which inhibit the action of acetylcholine, also inhibit the vasodilatation caused by adrenaline
- These substances, when perfused through the vessels, exert an action like that of ergotoxine or priscol, they reverse the constrictor action of adrenaline
- These substances can themselves exert a constrictor action on the vessels, and this constrictor action, like that of acetylcholine seen in the rabbit's ear vessels after 24 hours, is reversed under the influence of priscol
- In occasional fresh preparations, the injection of adrenaline regularly causes vasodilatation, though after several hours' perfusion with Locke's solution, the injection causes vasoconstriction
- The constrictor action of histamine in the rabbit ear vessels is abolished by the substances which abolish that of adrenaline Priscol does not reverse the action of histamine. In occasional fresh preparations, histamine causes vasodilatation
- The view that adrenaline has a dilator action of its own is discussed
- The view that adrenaline dilatation and constriction are produced at the same site is discussed
- The similarity in the action of adrenaline and acetylcholine is emphasized
- The evidence is considered to support the theory of drug action by competition, and to suggest the mechanism of adrenaline reversal

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ACTIONS OF SODIUM AZIDE

BY

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(Received July 23, 1948)

Hydrazoic acid or azoimide is a colourless liquid with explosive properties which solidifies at -80° C and boils at 37° C Azoimide gas has a characteristic pungent odour First prepared by Curtius (1890) it is a triazo compound in which a complex of three nitrogen atoms acts as a monad Hendricks and Pauling (1925) showed that the N₂ ion is constituted $N \rightleftharpoons N \rightleftharpoons N$ in chain Hydrazoic acid is a feebly dissociating Smith and Wolf (1904) showed that it was acıd toxic on inhalation by small animals, causing a fall in blood pressure, tachycardia, and stimulation of respiration, and that in solution it paralysed preparations of isolated frog muscle (1927) observed that the gas was a convulsant in frogs, and Hildebrandt and Schmidt (1937) extended the observation to cats and also noted a stimulant effect on gut Stern (1927) and Kocher (1930) described acute collapse in human beings from inhalation of the gas, and Graham, Robertson, and Rogan (1948) studied symptoms of hypotension in a group of workmen exposed to the fumes of azoimide intermittently over a period of years Hydrazoic acid forms explosive salts with heavy metals, the lead salt being used as a detonator Fairhall, Jenrette, Jones, and Pritchard (1943) have discussed the hazards of this substance as an industrial poison

The azoimide salt of sodium is stable, neutral and fully dissociated in solution (West, 1900) Loew (1891) published an account of experiments on the toxicity of sodium azide, in which he showed that approximately 40 mg/kg was lethal to mice, while 30 mg killed a dog in 104 min from respiratory failure and cramp. Smith and Wolf (1904) showed that sodium azide had the same effect as azoimide in lowering blood pressure stimulating respiration and increasing the rate of the heart. They found that the isolated perfused heart of the rabbit was inhibited by a concentra-

tion of 1 in 90,000 Further observations have been reported by Graham (1948)

Most recent-work with the azide radical has been confined to the investigation of the effect of sodium azide on cellular respiration. Keilin (1936), Keilin and Hartree (1934 and 1935), and Stannard (1939) have shown that azide interferes with cellular metabolism by inhibiting the oxidation processes in which cytochrome plays a part, it also inhibits indophenol oxidase and liver catalase, but not glycolysis. The salt is fungistatic and forms a compound with methaemoglobin in vitro but not in vivo.

Toxicity

'The LD50 in groups of white mice was found to be 28-34 mg/kg by intraperitoneal injection, 19 mg/kg intravenously, and 27 mg/kg by mouth The LD5 1p was 23 7 mg/kg The LD50 of sodium nitrite was 168 mg/kg and of potassium thiocyanate 600 mg/kg measured at the same time According to Fairhall et al (1943) the LD75 of NaN, for rats is 75 mg/kg 1p

Mice, rats, guinea-pigs, and rabbits injected with sodium azide by the oral, subcutaneous, intramuscular, intraperitoneal, or intravenous routes all showed similar symptoms, which varied only in degree and rapidity of onset with varying dosage levels of the salt Sublethal doses caused a preliminary stimulation of respiration and might give rise to clonic convulsions. This phase was accompanied by increased urination and passage of faeces and was followed by a prolonged period of collapse such as is seen with nitrite and thiocyanate Death occurred quietly with azide if the dose was such as to delay the termination for some hours, but if the dose was overwhelming death occurred acutely in convulsions. In these circumstances mice and rats assumed a characteristic death posture with the head flexed, the forelimbs flexed and pronated, and the tail and hind limbs extended

Cardiovascular System

Systemic blood pressure — Small doses of sodium azide caused a sharp transient fall in



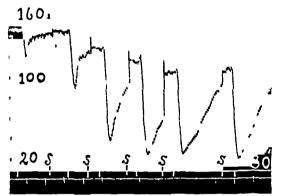


Fig 1—Cat 634 kg Chloralose, 80 mg/kg i v Upper line respiration (stethograph lever), next line carotid blood pressure, injection signal and drum stop (S), base line for zero blood pressure and time in 30 sec Transient fall in blood pressure and stimulation of respiration occurs on each injection of $2 \mu g$ NaN₃ at intervals of $5 \min$ The effect on respiration is minimal the effect on blood pressure increases on repetition

arterial blood pressure in cats and rabbits, the former being more sensitive than the latter. The amount of this fall depended on the initial level of the blood pressure, the anaesthetic used, and the dose of azide given. Cats were most sensitive

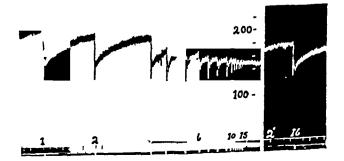
when anaesthetized with ether and given aqueous hydrazoic acid intravenously under these conditions a fall of blood pressure of about 60 mm Hg was observed with 0.5–1.0 μ g HN₂/kg was needed to produce a similar effect

If the dose of sodium azide given intravenously to cats was small (10 μg /kg or less) the response increased with repetition until it became constant, and the pressure did not return to its former level between doses (see Fig 1) If the initial dose was larger (or in rabbits) this early increase in response with repetition of doses was not seen

There was no diminution of response to repeated mjections of sodium azide (5-15 µg/kg) so long as the blood pressure was permitted to return to its initial level between doses time interval between administrations of the azide was shortened or the dose given at the same intervals of time was increased, the blood pressure did not return to its original level. In such circumstances the effects of repeated doses of azide decreased and might be extinguished As soon as time was given for restoration of the pressure approximately to its initial level the full effect of the original dose of azide was restored relationship between the initial level of the blood pressure and the response is illustrated in Fig 2

The fall in blood pressure resulting from administration of small doses of azide to cats or rabbits probably results from a direct action on the smooth muscle of the blood vessels occurred in anaesthetized eviscerated animals after atropine and vagotomy, after benadryl, and after excision of the carotid sinuses and adrenalectomy, and in spinal cats similarly prepared The effect was not modified by previous injection of eserine or nicotine Records of the volume of the spleen and foreleg showed a decrease in the volume of both accompanying the fall in pressure, but in three preparations out of ten the spleen dilated actively Perfusion of the hind limbs of the rat through the aorta with a saline solution

FIG 2—Cat \$3 2 kg Chloralose, 80 mg/kg 1 V Upper line carotid blood pressure, middle line time in 2 min, lower line zero blood pressure and injection signal 50 μg NaN₃ in 0.25 ml water was injected quickly at each signal Nos 1–3 were given at 15 min intervals, nos 4–6 at 5 min intervals nos 7–10 at 2 min intervals, nos 11–15 at lesser intervals Between nos 15 and 16 an interval of 45 min. The degree of response to azide is related to the initial level of blood pressure.



under constant pressure and recording of the volume of the venous effluent showed that azide increased the rate of flow as a result of vasodilatation

If the dose of sodium azide injected into cats was increased to 10-20 mg/kg a rise in blood pressure occurred in the majority of animals, though some reacted with a severe and prolonged fall in pressure. The rise in blood pressure usually obtained was not modified by excision of the carotid sinus area or vagotomy. It was abolished by previous excision of the adrenal glands or by blocking agents such as dibenamine and dihydroergotamine. That the stimulation of the adrenals is an indirect one was proved by failure to repeat the effect after bilateral splanching nerve section and by the failure to obtain

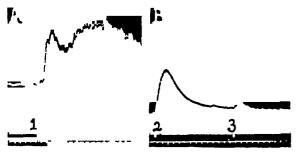


Fig 3—Cat δ 3 2 kg Ether, chloralose 80 mg/kg Upper line carotid blood pressure, middle line zero blood pressure and injections, lower line time in 10 sec Preceding the records shown the cat received 10 mg NaN₃/kg i v (with effect as in A), and bilateral excision of the carotid sinuses At 1 repeat of 10 mg NaN₃/kg giving prolonged rise in blood pressure Between A and B bilateral splanchnic nerve section At 2 adrenaline (2 μg/kg) At 3 repeat 10 mg NaN₃/kg with no effect

the effect in spinal cats (see Fig 3) A similar rise in pressure was observed in rabbits after similar doses of azide

The heart—Injection of $50 \mu g-10$ mg sodium azide into the inflow of the perfused heart of the cat (Langendorff preparation) caused an increase in the force but not in the rate of contraction 2–5 mg caused transient inhibition of some preparations. In the isolated rabbit heart the increase in force was less marked and inhibition was more often seen with the higher doses. In both species dilatation of the coronary vessels with increase in coronary flow occurred. Fig. 4 shows the effect of a dose of $50 \mu g$ sodium azide on the perfused cat heart and the coronary flow from it. In view of the hypotensive effect of sodium azide on systemic blood pressure the action of other agents with a similar hypotensive activity was tested on

the coronary circulation The dilator effect of an equimolar amount of sodium nitrite was less intense but longer in duration than that of 50 μ g sodium azide, potassium thiocyanate had no effect (see Fig. 5)

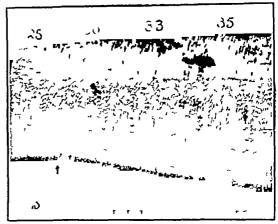


FIG 4—Record of the action of a perfused cat heart (Langendorff preparation) Time in 10 sec. The figures at the top are a measure of the coronary flow in ml /min. At the arrow 50 μg. NaN₂ was injected into the perfusing cannula

The effect of intravenous injection of sodium azide on the heart of anaesthetized cats and rabbits was variable. In rabbits anaesthetized with urethane $10-100~\mu g$ of azide increased the ferce and sometimes the rate of contraction of the heart, but in the majority of animals the heart was temporarily slowed. Atropine or vagotomy abolished this slowing but left the increase in force

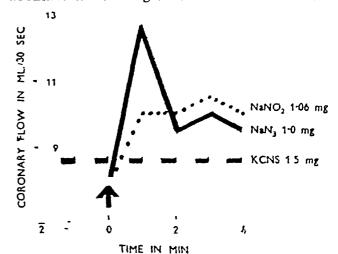


Fig 5—Graph of the coronary flow in the isolated perfused heart of the cat (Langendorff preparation) At intervals of 20 min equimolar amounts of potassium thiocyanate, sodium nitrite, and sodium azide were injected (arrow) KCNS had no effect (interrupted line), NaN, had a powerful but short-lasting dilator effect (solid line), and NaNO, had a longer-lasting effect (dotted line)

unaffected Larger doses of azide (5-10 mg of azide/kg) produced a marked initial slowing and inhibition of the heart followed by increase in cardiac activity above the initial level. This slowing was abolished by vagotomy or atropine, leaving the stimulation unaffected. In cats the effects of azide were similar, but this animal was more sensitive to sodium azide than the rabbit. A few μ g of azide per kg brought about a marked increase in the rate and force of the heart (see Fig. 6) larger doses (5 mg/kg) caused a transient inhibition, abolished by atropine or vagotomy and unobtainable in the spinal cat, followed by a prolonged increase in the rate and force of the heart.

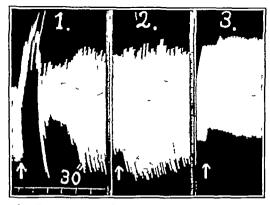


Fig 6—Direct myocardiograph tracing from cat 3 2 kg, ether and 80 mg chloralose/kg 1 Sodium azide (5 mg/kg) transient inhibition followed by stimulation 2 Repeat after bilateral cervical vagotomy and 1 mg atropine sulphate/kg, followed by 15 mg dibenamine HCl/kg stimulation without preliminary inhibition 3 Repeat after bilateral adrenalectomy absence of stimulation

not affected by dibenamine (15 mg/kg) but prevented by previous adrenalectomy (see Fig 6) In a certain number of these animals, as already mentioned in the section dealing with the effects of azide on the blood pressure, the effect of large doses of the salt were mostly inhibitory with a fall in pressure replacing the rise in pressure. In these circumstances the cardiac action was also inhibited for a long period after injection, and adrenalectomy made little or no difference to the reactions obtained. Excision of the carotid sinus area did not modify these responses

Since sodium azide in small doses appeared to have a more powerful effect than the other commonly used hypotensive agents on the blood flow in the systemic and coronary circulations comparison was made of their activities on the heart of the whole animal under light anaesthesia (ether, chloralose or urethane) As is shown in Fig 7 the

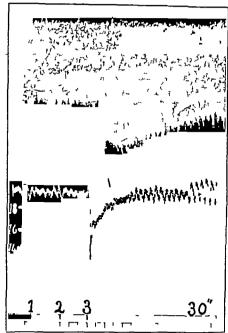


FIG 7—Cat 3 4 kg, 80 mg chloralose/kg 1 v Upper line direct myocardiograph lever, next line carotid blood pressure, injection signal, time in 30 sec At 1, 2, and 3 injection of equimolar amounts of KCNS, NaNO₂, and NaN₃ (50 µg) respectively Note transient inhibition of heart followed by augmenta tion of the beat and abrupt fall in blood pressure after injection of azide

characteristic cardiovascular action of azide—stimulation of the heart and fall in blood pressure—is obtained with amounts of azide such that equimolar amounts of nitrite and thiocyanate have no obvious effect

Respiration

The rate and depth of breathing were increased by inhaled hydrazoic acid gas in very low concentrations (see Graham, Robertson, and Rogan, 1948, Fig 1) and by injected azide in all doses With doses of less than 1 μ g sodium azide per kg the stimulant effect on respiration did not last so long as the accompanying fall in blood pressure The degree of stimulation might be such as to cause marked irregularity of breathing and even With suitable generalized clonic convulsions doses these convulsions appeared immediately after injection of the azide and were not asphyxial in origin, but doses of azide insufficient to give rise to a convulsive response might, after a variable period of stimulation and irregularity of breathing, bring about a depression and gradual failure of respiration, this was often complicated by terminal asphyxial convulsions

Other actions

Sodium azide dilated the perfused bronchus of guinea-pigs and stimulated both isolated and intact intestine of rabbit, guinea-pig, and cat In anaesthetized animals this stimulation of the gut, accompanied by flushing with arterial blood, was obvious records of the movements of an intestinal loop and of the pressure within it revealed a transient relaxation of the muscle preceding the increase in activity The isolated uterus of rat, rabbit, and guinea-pig was not affected by azide, but that of the cat was stimu-In the pregnant cat under ether, however, this effect was negligible with amounts of azide which caused a prolonged fall in blood pressure the urinary bladder contracted vigorously and expelled urine There was no difference in the diuretic response of groups of rats injected with small doses of azide after receiving water by mouth and control animals not given azide, but if the dose of azide was increased the rats showed evidence of collapse and urine production ceased Frequency of micturition as seen in intact animals given azide is not due to diuresis but to the action on the urinary bladder, probably a direct one Azide is water soluble and was absorbed from all routes of administration. It did not discolour the blood with methaemoglobin as did nitrite like nitrite it was not excreted unchanged in the urine, which failed to give the brown colour reaction with ferric chloride obtained in vitro with dilute sodium azide before injection or feeding

DISCUSSION

The actions of sodium azide on the cardiovascular system of mammals are similar to those of sodium nitrite The effect of inhaled amyl nitrite on the systemic blood pressure as described by Bradford and Dean (1894) is similar to that of hydrazoic acid gas as described by Smith and Wolf (1904) and elaborated by Graham, Robertson, and Rogan (1948) That the tachycardia caused by nitrite is a reflex one following upon the fall in blood pressure was shown by Dossin (1911), whose findings with the action of nitrite on the isolated perfused heart are paralleled by those described above for azide (1904) showed that nitrite is a coronary dilator, and Boyer, Wégria, and Green (1939) and Essex Wégria, Herrick and Mann (1940) confirmed this finding by modern techniques The same action has been demonstrated with azide Both nitrite and azide stimulate the respiration, but Heymans Bouckhert, and Dautrebande (1931) attribute to the carotid sinus a larger part in the reflex stimulation of respiratory and cardiovascular mechanisms by nitrite than the present work indicates is played by the carotid sinus in the action of azide Leech (1893) and Smith and Wolf (1904) have shown respectively that nitrite and azide inhibit the contractile power of isolated frog gastrocnemius muscle According to Beams and Barlow (1932) nitrite causes a contraction and then a relaxation of isolated strips of rabbit gut in Locke's solution azide causes a contraction in vitro and a relaxation followed by a contraction Both substances dilate the perfused in vivo Both are modified by metabolic probronchi cesses in the body their fate is as yet unknown

The main differences lie in the greater direct effect of azide on the central nervous system, especially on the respiratory centre, the more powerful action of azide on the peripheral vascular bed, the absence of formation of methaemoglobin in the blood stream of animals receiving azide intravenously, and the proven effect of azide on various enzymatic processes in the living cell

In human beings it has been shown that hydrazoic acid fumes lower systolic and diastolic blood pressure to a profound extent for some three to six hours with the production of a mild degree of headache

The mechanism of action of azide is complex Peripherally it relaxes the smooth muscle of blood vessels and bronchi while increasing the force of the cardiac contraction This results in a greatly increased coronary flow accompanying a fall-in systemic blood pressure Centrally it stimulates the medulla, which results in a stimulation of respiration, an initial vagal inhibition of the heart, and a subsequent sympathetic stimulation of the Azide also has a direct stimulant action heart on cardiac muscle which may play a part in this phenomenon The resultant effect of these opposing forces is determined by the species and state of the animal, especially the initial blood pressure. the sensitivity of its nervous system and reflex mechanisms, the anaesthetic administered, and the dose and rate of administration of the azide. The larger doses of azide (mg rather than μg /kg) powerfully affect the sympathetico-adrenal mechanism and cause a prolonged rise in systemic blood pressure and a great increase in cardiac force, probably brought about by a release of adrenaline from the suprarenal glands variable effects on the volume of the spleen and the leg are the result of these conflicting mechan-The activity of gut and bladder is increased, that of the uterus is little altered Stimulation of the central nervous system, which may be severe

enough to cause a characteristic convulsive seizure 13 in other cases followed by depression leading to asphyxia from respiratory failure

SUMMARY

Sodium azide, a neutral stable salt of hydrazoic acid, is a potent hypotensive agent which dilates peripheral blood vessels by direct action stimulates cardiac muscle and dilates the coronary vessels directly

It affects the rate and force of the mammalian -heart in vivo by stimulating the vagal and sympathetic cardiovascular mechanisms such effects are produced centrally rather than by carotid sinus reflexes

It stimulates respiration and in large doses produces generalized convulsions followed by respiratory depression

It increases gut and bladder contractions but hardly affects the uterus

The general action is similar to that of sodium nitrite, but azide is more powerful

The LD50 1 p in white mice was 28-34 mg/kg when that of sodium nitrite was 168 mg/kg

It does not produce methaemoglobin in vivo and is excreted as a metabolite which no longer gives a brown colour reaction when ferric chloride is added to the urine

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A KYMOGRAPHIC STUDY OF THE ACTION OF DRUGS ON THE LIVER FLUKE (FASCIOLA HEPATICA)

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The difficulty of culturing parasitic worms in vitro remains the major limiting factor in the development of methods for studying anthelmintic* activity directly on the parasite. This is because of the difficulty of maintaining in vitro a suitable environment for the different parasites This has been achieved in a partial sense by Chu (1940) for Chlonorchis sinensis and Schistosoma japonicum, by Lamson and Brown (1936) for Ascaris, and by Smyth (1948) for plerocercoid larvae of Schistocephalus solidus and Ligula intesunalis, but for many other parasites methods for obtaining the same conditions do not yet exist In their search for material which might be likely to provide alternative means, the early workers neglected the phylogenetic relationships of the different parasitic worms. Two main groups of helminth parasites exist the nemathelminthes and the platyhelminthes

Owing to the work of Robello and Rico (1926) and Baldwin (1943), a method exists for the *in vitro* study of anthelminuc activity on nematodes. This method, moreover, has enabled a start to be made on the mode of action of drugs on nematodes. Von Schroeder (1885) and Trendelenberg (1916) both reported that santonin, which has a high therapeutic reputation as an anthelminuc against nematodes, appears to have no effect on the activity of the whole worm, when it is tested by observing the activity of whole ascaris *in vitro*. Baldwin, however has suggested it may bring about incoordination of movement by virtue of a simultaneous stimulation of the body musculature.

(intermediate preparation) and depression of central nervous control (anterior preparation) Many a priori reasons exist for considering the effect of substances on the movement of the parasite to be a primary mode of action. Thus we have chosen to study the effect of drugs on the liver fluke as a representative platyhelminth worm by a kymographic technique of short duration. The present paper is, therefore, concerned with a method for studying the mode of action of drugs on trematodes, and the value of the liver fluke (Fasciola hepatica), as a preparation for screening substances for possible anthelmintic activity against the parasitic members of both phyla, is assessed

Colupoun of parasites

The flukes were obtained from the bile ducts of bovine livers and were dissected out within half an hour of the death of the host before the liver cooled to room temperature. They were washed and subsequently placed in boiling-tubes containing Ringer's solution buffered (to pH 8.5) at 37° C, not more than two flukes to each tube. This was found necessary in order to prevent the flukes from attacking each other, which they did when large numbers were enclosed together. Preparations were made from these flukes within six hours of collection. Satisfactory flukes always showed rippling movements of the whole body, and movement by means of the suckers for at least 24 hours.

Methods of recording movement

Kymograph tracings were made of the movements of a fluke suspended in Ringer's solution at 37° C the fluke being under slight tension. The attachment for the recording arm and fixed point were by means of platinum hooks passed through the body wall posterior to the ventral sucker and close to the

^{*}The term anthelmintic is used by us to indicate that the drug has been found effective for the cure of infestations of helminth parasites either in veterinary or clinical practice

posterior end of the body. It was found essential to keep the fluke below the surface of the Ringer's solution while these attachments were being made in the shortest possible time. The fluke was then allowed 10 minutes to recover before recording was started, after which normal movements were recorded for 15 minutes.

Method of testing drugs

Drugs soluble in water were dissolved in Ringer's solution, those which were precipitated in this medium were separately dissolved in water to which the saline constituents were then added as this delayed precipitation for long enough to make the test (santonin. kamala, umbelliferone, and coumarine) drugs were emulsified by the method of Baldwin The substances were tested initially at a high concentration (1 1,000) and, if active, at lower concentrations suggested by the response obtained Finally the minimal effective concentration, as judged in most instances by the disappearance of activity within 45 min after the addition of the drug, was obtained with at least four flukes. The absence of effect at 1 1.000 on at least two preparations was taken to indicate that the drug was without activity

RESULTS

Rhythmical activity was found in a high proportion of worms taken from bovine livers, but in general worms from sheep's livers could not be relied upon to give good rhythmical activity Rhythmical activity, which was occasionally interrupted by short quiescent periods, showed a marked variation in amplitude from worm to worm, but, after the establishment of the rhythm in any one preparation, the amplitude and the tone remained approximately constant for at least 2 hours (Fig. 1)

Three types of drug activity have been and stimulant, paralysant, and lethal

Stimulant drugs

On addition of the substances with stimulant properties listed in Table I, some interference with the activity of the preparation occurred, and subsequently more rapid rhythmical movements were

TABLE I

1 171107	Nature of preparation Solution Emulsion Solution Emulsion Solution	Effective concentration 1 2,000-1 4,000* 1 2,000-1 5,000* 1 20,000-1 5,000* 1 10,000-1 5,000*
Tetrachlorethylene Hexachlorethane β-Naphthol p-Cymene	Emulsion Solution Emulsion	1 2,000-1 5,000* 1 20,000-1 5,000* 1 10,000-1 5,000* 1 4,000*
Strychinder ICI Coumarine Umbelliferone β-Phenylethylamine HCl Ephedrine HCl Tyramine HCl Amphetamine sulphate l-Amphetamine sulphate d-Amphetamine sulphate	;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;	1 10,000 1 2,000 1 2,000 1 5,000 1 1,2,000 1 1,000 1 20,000-1 10,000 1 10,000 1 80,000

^{*} Indicates that the drug possesses other types of action at higher concentrations or after acting for longer periods of time

resumed with or without a change in amplitude and tone There was also a marked difference in The two lactones. the duration of these effects coumarine and umbelliferone, produced rhythmical movements of large amplitude and low frequency at low concentrations These movements, however, gradually diminished within a period of half an hour, and were replaced by sustained when high concentrations were The stimulation produced by strychnine tested hydrochloride was characterized by increase in the frequency, reduction in the amplitude, and increase in the tone The four amines all produced an increase in the amplitude and frequency after the initial contraction had subsided and produced some increase in the tone

The chlorinated hydrocarbons with known anthelmintic potency and therapeutic value against

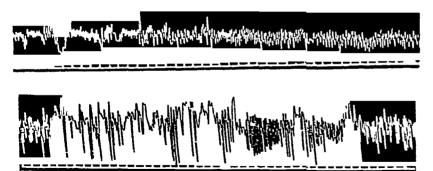


Fig 1 — Two representative records of normal movement (In all tracings upward stroke represents contraction Time markings in minutes)

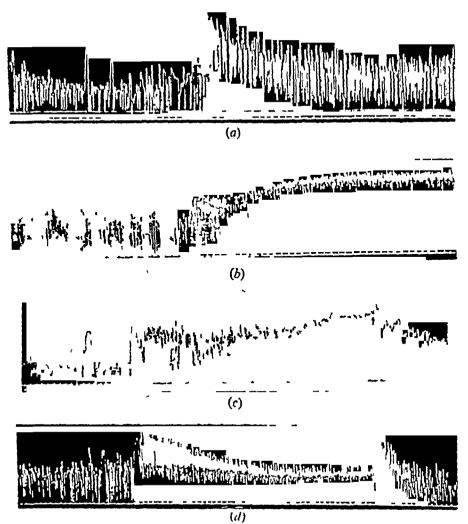


Fig 2 —Stimulant drugs Action on normal movement of (a) amphetamine (1 1,000), (b) tetrachlorethylene (1 5,000), (c) hexachlorethane (1 20,000) followed by amphetamine, (d) coumarine (1 1,000) followed by amphetamine

infestations of the liver fluke all possess stimulant properties at low concentrations. This property was exhibited to a more marked degree by hexachlorethane (Fig. 2)

From the records it will be seen that the amines have a pronounced potentiating action on the normal rhythm, increasing both the amplitude and

the frequency with only slight effect on the tone of the muscle Moreover, amphetamine was the most powerful of these potentiating amines

Paralysant drugs

Early on in the investigation it appeared likely that amphetamine might be used to bring back

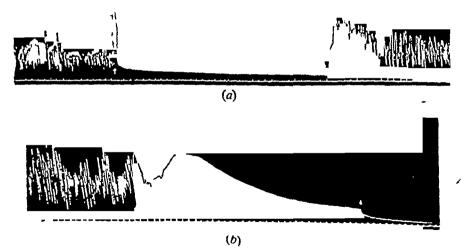


Fig 3—(a) Paralysant action of pelletierine tannate (1 1,000) followed by response to amphetamine (b) Lethal action of hexylresorcinol (1 5,000) followed by absence of response to amphetamine

TABLE II

DRUGS WHICH PARALYSE THE PREPARATION BUT RHYTH-MICAL CONTRACTION IS RESTORED BY AMPHETAMINE 1 5,000

Drug	Nature of preparation		Effective concentration
Nicotine Carbaminoylcholine chloride (Doryl) Sodium amytal Arecoline HBr Oil of chenopodium Pelletierine tannate Phenylurethane Santonin Kamala	Solution ,, ,, Emulsion Solution Emulsion Filtered saturated solution	1 1 1 1 1 1 1 1 1 1	200,000 20,000 5,000 10,000,000 20,000-10,000* 2,000 2,000* 1,000

Indicates that the drug possesses other types of action at higher concentrations or after acting for longer periods of time

TABLE III

DRUGS WHICH PARALYSE THE PREPARATION AND RENDER IT INSENSITIVE TO THE ACTION OF AMPHETA-MINE 1 5,000

Drug	Nature of preparation	Effective concentration
Oil of chenopodium p-Cymene Thymol Hexylresorcinol β-Naphthol Carbon tetrachloride Tetrachlorethylene Hexachlorethane Ext. filix mas Gentian violet Phenylurethane Chlorbutol Kamala	Emulsion Solution "" Emulsion "" Solution "" Sat solution	1 5,000 1 1,000 1 10,000 1 10,000-1 5,000 1 4,000-1 2,000 1 1,000 1 1,000 1 1,000 (see text) 1 5,000 1 5,000 1 1,000 1 1,000 1 1,000

normal rhythmical activity to preparations when the movement had been reversibly altered by a drug, this might occur after inactivation by paralysant drugs or when the type of movement was markedly altered by other stimulant drugs No súch action would be expected after an effect which was to any extent irreversible, or after lethal drugs It was therefore decided to add amphetamine sulphate in a concentration of 1 5,000 to the bath in the absence of the test substance, whenever the activity of the preparation had been obliterated or altered at the end of 45 min. This was done by replacing the solution of the test substance by Ringer's solution containing 1 5,000 amphetamine sulphate By this means it was found possible to distinguish between drugs with paralysant action (listed in Table II, Fig. 3) and drugs with a lethal or probably lethal action (listed in Table III, A large number of miscellaneous substances with no known anthelmintic action were tested and found to have no more than a transient action (Table IV, Fig 4) The fluke musculature is relaxed to different degrees by parasympathomimetic drugs, except pilocarpine, and this relaxation is antagonized by amphetamine

TABLE IV

DRUGS WHICH FAILED TO CAUSE COMPLETE PARALYSIS OF THE PREPARATION UP TO A CONCENTRATION OF 1 1,000 (MINIMUM 45 MIN)

d-Tubocurarine chloride Phenothiazine Sodium tauroglycocholate—Yohimbine HCl Ethyl alcohol (1%) Sulphanilamide Sulphathiazole Penicillin (1,340 i u /c c) Neosalvarsan Emetine HCl Acetylcholine chloride Acetyl-β-methylcholine chloride* Pilocarpine nitrate Eserine sulphate*

Atropine sulphate Hyoscine HBr Histamine acid phosphate Cocaine HCl Oumne sulphate Morphine HCl Caffeine Guanidine HCl Sodium bromide Phenyl urea

* Transient depression

Lethal drugs

The chlorinated hydrocarbons which in low concentrations stimulate the fluke are lethal at concentrations ranging between 1 5,000 and Hexachlorethane has exceptional prop-1 1,000 erties which make it difficult to obtain known concentrations of it above 1 5,000, it is insoluble in water and sublimes below the boiling point of Thus, although sufficient substance was added to make an emulsion at 1 1,000 with alcohol some of the substance was precipitated The emulsion made in this way, however, obliterated all movement in 90 min, after which amphetamine was without effect. The 1 5,000 solution



Fig 4 -Absence of effect of morphine sulphate (1 1 000)

almost completely eliminated the activity, and correspondingly its action was only slightly affected by amphetamine. It would therefore appear to be a lethal drug in low concentrations, possibly with greater effect than the other chlorinated hydrocarbons. Ext. filix mas is lethal at the same concentration in a shorter time. This is also true of gentian violet.

Ineffective substances

Phenothiazine was the only inactive anthelminute with the exception of drugs active on blood flukes

DISCUSSION

The value of any particular approach to the study of anthelmintics can best be understood when it is realized that the host-parasite relationship is a special instance of the organismenvironment relationship which forms the background to all biological studies practice possesses a variety of methods for combating parasitic infestations which act in one of four ways on the biological system comprising the host-parasite relationship The first is by alterations in the relationship of the host to its environment, the second is by effecting changes in the internal environment of the host, the third is by affecting the relationship of the parasite to the internal environment of the host, and the fourth is by altering the internal environment of the parasite itself. The pharmacology of anthelmintics should be concerned with all four divisions, but in practice the first division is usually considered a separate field of study in the domain of ecology Within the three remaining fields which are concerned with the administration of substances to infested animals or man the study of anthelmintics has been approached either as a chemotherapeutic problem or as if it were a pharmacological study on isolated organ systems of vertebrates both aspects must be integrated to provide a satis factory approach to the problem Chemotherapeutic tests are primarily effective for the detection of substances of therapeutic value, because microorganisms are primarily dependent on a continuous process of reproduction for their distribution and for the location of the reproductive phase of their Substances, therefore, which interfere with metabolic processes by whatever means are effective in reducing the distribution and influencing the location of micro-organisms In parasitic worms, however, the distribution and the location of the adult reproductive worm are differentiated functions and depend on the separate physiological processes of reproduction and movement Distribution is largely dependent on reproduction though movement plays some part in it, but the location of the adult is almost wholly dependent on movement which appears to be influenced by environmental factors. Elimination of the parasite, therefore, is dependent upon the drug affecting either the mechanism by which location is achieved and maintained or by suppressing reproduction. It is at present impossible to study the effect of drugs on reproduction in vitro because of difficulties of culturing them, but certain pharmacological techniques have been applied to the study of movement in parasitic worms, as in the present paper

We require to know the effect of any drug on the components of the fluke's movement and on the co-ordination of these in its behaviour. Provided that the same effects are produced in vivo as in vitro we can then hope to get some idea of the possible modes of action of anthelmintics which affect movement. At present, we have only taken a step in this direction. The kymographic technique, which we have used, has enabled us to distinguish three different types of action affecting movement from which a classification of drugs can be made.

Some drugs are stimulants as defined earlier these are the amines, the lactones, and strychnine Other drugs are stimulants at low and lethal at higher concentrations—namely, the halogenated hydrocarbons, p-cymene and β -naphthol. This combined action may well be the reason why the halogenated hydrocarbons are effective on the liver fluke itself in vivo, whereas gentian violet, which in vitro is more lethal but has no stimulant action, is ineffective *

Tetrachlorethylene has been shown by Rogers (1944) to have a stimulant effect on Nippostrongy-lus muris, this appears to be an exaggeration of normal movement since it caused the parasite to leave the mucous lining of the intestine. Halogenated hydrocarbons, therefore, as a group are likely to be stimulant to both phyla. Baldwin's (1943) technique using segments of Ascaris would appear to be inadequate, therefore, to reveal all types of stimulant action probably because his preparations are only part of the whole animal

Finally there are drugs with paralysant action at low concentrations which become lethal as the concentration is raised—e g, phenylurethane and oil of chenopodium

Because major systematic differences such as those between phyla are most likely to be associated

with major biochemical differences it is to be expected that comparison between members of the two differential phyla will reveal the presence or absence of selective* action within the helminths when tested *in vitro* It is therefore worth while comparing our results with those of Baldwin

As might be expected, the liver fluke is killed by all the protoplasmic poisons which affect Ascaris Moreover, such comparison demonstrates that the liver fluke is affected by lower concentrations of the drugs which are lethal to both preparations The liver fluke is also affected by some drugs which are not effective on Ascans-1 e, the fluke preparation is sensitive to a wider range of known anthelmintic drugs than the Ascaris preparation should here be noted that Baldwin's preparation is insensitive to the action of gentian violet, which is active on threadworms in vivo This may be due to an m vitro selective action of gentian violet on threadworms, but it might equally well be due to the fact that Baldwin's preparation does not allow direct access of the substance to the gut of the Ascaris preparation The evidence available suggests that the cuticle is a more effective, and probably also a more selective, barrier to the penetration of drugs in Ascaris than in the liver fluke Comparative tests on flukes with intact cuticles. and cuticles pierced at the point of attachment, reveal that the intact fluke is only slightly more resistant to all the drugs which we have tested On the other hand some drugs are without effect on Ascans because of fundamental differences in the neuromuscular mechanism. An explanation of the different responses to pelletierine and arecoline should be sought in one or both of these mechanisms By contrast, those drugs which are effective for the elimination of cestodes from the intestine in vivo are also active on the liver fluke in vitro, a result which is consistent with the systematic relationships of the two parasites paralysant action of arecoline, which relaxes the muscle, parallels the results obtained by Betham (1946) on segments of Taenia

The kymographic test of Betham does not allow any strict comparison to be made between the action of drugs on trematodes and cestodes, owing to the restricted use to which it was put. The only drug used as an anthelmintic with selective action directly on nematodes is santonin. Pelletierine and ext filix mas because they are active only on the fluke in vitro but not on Ascans and because

they are used exclusively against cestodes in the intestine, are likely to prove selective for platy-helminthes. Gentian violet, on the other hand, although it does not affect Baldwin's preparation, does eliminate nematodes (threadworms) from the intestine and is therefore almost certainly active on both phyla. In this connexion it should be noted that, of the two lactones which are stimulant to the fluke, coumarine paralyses Ascaris (posterior segment) and umbelliferone is without effect. This is also true of strychnine. The mode of action of phenothiazine and drugs active on blood flukes is still obscure

Stephenson (1947) measured the effect of carbon tetrachloride, tetrachlorethylene, and gentian violet on the survival time of the liver fluke in vitro and was unable to detect any action of the chlorinated hydrocarbons, but obtained some effect with gentian violet, thus demonstrating that his method was less sensitive than the one under discussion We attribute this to the difference in viability of the preparation in the two types of test acute test, using as a measure of activity the effect on movement, the viability of the preparation is This is not so in a test based on survival time, as the viability of the preparation falls off rapidly towards the end of the experimental period when the preparation is required to be most sensitive to differences in activity of the drugs under Moreover, the method led to paradoxical results in the hands of Chu (1940), who showed that Chlonorchis sinensis survived longer in higher concentrations of certain drugs (e.g., methylene green and trypan blue) than at lower concentrations that in effect there was a reverse relationship between dose and effect which was attributed to a reduction in oxidation rate preventing the parasite in vitro from poisoning itself with its own excreta

The comparisons we have made suggest that the majority of drugs used as anthelmintics are proto plasmic poisons acting on both phyla and also on The apparent selectivity in the anthelmintic activity of the protoplasmic poisons in vivo does not therefore reflect a selective action on different parasites but rather the importance of different biochemical and physiological conditions in the immediate neighbourhood of the parasite affecting the action of these poisons This emphasizes the need for tests capable of distinguishing the selective action of drugs in vitro if safer anthelmintics are to be found. This does not mean that we require to test drugs on isolated members of each species but that we require to assess the significance of systematic differences revealed by tests on representative members of each phyla

^{*}The word selective is used by us to indicate that drugs act exclusively on a few parasites. It is qualified by the adverbs in vitro and in vivo (Specificity should be reserved for testing the action of drugs on different parts of an organism. In this way its use in this field is consistent with the rest of pharmacology)

SUMMARY

- 1 It is possible to obtain rhythmical kymographic records from fresh bovine flukes suspended in Ringer's solution at a pH range from 65-85. These movements are maintained for a period of at least 2 hours and frequently as long as 6 hours.
- 2 The effect of known anthelmintics and of a number of other drugs, particularly those affecting the neuromuscular mechanism of vertebrates, have been tested on this preparation by allowing them to act for a maximum period of 45 min
- 3 At the end of this period the addition of amphetamine restores rhythmical activity provided the drug has had only a paralysant action on the fluke. In this way it has been possible to distinguish between stimulant, paralysant, and lethal drugs
- 4 It is suggested that the possible reason why the chlorinated hydrocarbons are so effective as anthelmintics against Fasciola hepatica is that they combine the stimulant action at low with the lethal at higher concentrations. A number of other anthelmintics have been shown to have other types of combined action
- 5 Comparison with the similar test on Ascaris segments (Baldwin, 1943) reveals that the liver fluke as a representative of the platyhelminthes is sensitive to all the drugs which affect Ascaris and in addition to umbelliferone, pelletierine, extract

filix mas, and gentian violet The significance of this comparison is discussed

We wish to thank Professor Frazer for his interest and valuable criticism, Dr E L Taylor, of the Ministry of Agriculture and Fisheries, Weybridge, for tests on gentian violet in fluke-infested rabbits, and the Veterinary Department of the City of Birmingham Meat Market, for their interest and co-operation in obtaining satisfactory supplies of parasites We also wish to thank the Egyptian Educational Bureau in London for financial assistance We gratefully acknowledge the receipt of substances used in the investigation from the following firms Dr M L Tainter, of the Sterling Winthrop Research Institute, Glaxo Laboratories Ltd, Greenford, Labaz Ltd, Brussels, Belgium, T and H Smith Ltd, Edinburgh, W J Bush and Co, Ltd, Imperial Chemical Industries Ltd, May and Baker Ltd, Savory and Moore Ltd

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SOME PHARMACOLOGICAL ACTIONS OF PALUDRINE

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Since the discovery of paludrine by Curd, Davey, and Rose (1945), few workers have studied its general pharmacological properties, though its lowtoxicity in comparison with many antimalarials is well known Spinks, Tottey, and Maegraith (1946) found that the absorption, distribution, and excretion of paludrine in the rat resembled that of mepacrine Spinks (1947) investigated the fate of paludrine given to rats and mice by mouth and found that it was rapidly absorbed from the stomach, a low recovery from the faeces and urine suggested that it was broken down in the Butler, Davey, and Spinks (1947) showed that the acute toxicity of paludrine varied both with the species and the method of administra-The intravenous or intraperitoneal injection of paludrine into rats and mice was followed by delayed deaths, the animals dving up to 24 hours after the injection, this was the more surprising in view of the fact that a closely related substance, 4430, did not exhibit this property. At first it was thought that these delayed deaths were caused by unusually prolonged retention of paludrine in the blood, but this was later disproved by measuring blood concentrations Chicks given paludrine intravenously died within 15 min. or not at all, and from this and other evidence it was concluded that the metabolism of paludrine in chicks (and probably man) was different from that in rats and mice

Hughes and Schmidt (1947) and Hughes, Schmidt, and Smith (1947a, b) also studied the absorption, toxicity, and excretion of paludrine in various animals. In the dog, paludrine induced copious salivation, loss of appetite, extreme cachexia, and cardiac arrhythmia Feeding tests were performed in which paludrine was mixed with the food the dogs refused to eat, and died from starvation Chen and Anderson (1947) investigated the toxicity and then described in more detail the effects of paludrine in the body In the anaesthetized or pithed cat, paludrine caused a temporary fall of the blood pressure The respiratory rate was increased but the depth

diminished Isolated loops of rabbit intestine were relaxed by paludrine and the isolated uterus of the guinea-pig was stimulated Contractions of isolated intestine of the guinea-pig, induced by histamine, were inhibited Innes (1947) showed that intramuscular injections of paludrine lactate caused necrosis, haemorrhage, oedema, and inflammatory exudate, with local involvement of nerves and vessels, this was most intense about 12 days after the injection It is now generally believed that some metabolic product of paludrine is responsible for its antimalarial activity (Hawking and Perry, 1948), but so far attempts to identify this product have failed

EXPERIMENTAL RESULTS

Toxicity — The acute intravenous toxicity of paludrine in mice of weights between 18 g and 25 g was studied The LD50 was found to be 22 mg/kg, which agrees well with the results of other workers As Butler, Davey, and Spinks (1947) observed, the toxicity of paludrine was sometimes delayed, and the figure for the LD50 given above was calculated after observing the mice for 72 hours after the injection A possible explanation of the delayed toxicity was that paludrine interfered with some esterase mechanism in the animal Blaschko, Chou, and Waida (1947) showed that paludrine had an affinity for pseudocholinesterase and for the benzoylcholinesterase of guinea-pig's liver

It was thought that the simultaneous injection of prostigmine, a very strong inhibitor of cholinesterase, might significantly alter the toxicity of The intravenous toxicity of prostigmine in mice was therefore determined, and from the dose/response curve two doses of prostigmine were chosen, one which killed no mice (90 µg/kg) and one which killed about 30 per cent of the mice (180 µg/kg) Each of these doses was then injected simultaneously with the LD50 dose of paludrine (22 mg/kg) The results

are summarized in Table I

TABLE 1									
INTRAVENOUS TOXICITY OF PALUDRINE, PROSTIGMINE, A	AND	MIXTURES	OF	THE	TWO				

Group	Dose of drug	No of mice used	Instant deaths	I	Oclayed dea	Total deaths	% Mortality	
				0-24 hr	24-48 hr	48-72 hr		
A	Prostigmine 90 μg./kg	15	0	0	0	0	0	o
В	Prostigmine 180 µg /kg	30	8	0	0	0	8	26 6
С	Paludrine 22 mg /kg	30	0	8	4	4	16	53 4
D	Paludrine 22 mg/kg + Prostigmine 90 µg/kg	30	8	5	5	3	21	70
E	Paludrine 22 mg /kg + Prostigmine 180 μg./kg	30	29	0	0	0	29	967

Table I shows that the injection of prostigmine simultaneously with paludrine increased the immediate toxic effect. The immediate deaths, taken as a percentage of the total deaths, were increased from 0 per cent to 38 per cent by the addition of a non-toxic dose of prostigmine (90 μ g/kg), and to 100 per cent by a dose of prostigmine (180 μ g/kg.), which alone killed about 27 per cent of animals

Effect of paludrine on the cardiovascular system

Action on blood pressure and vessels—In the anaesthetized animal an intravenous injection of paludrine usually caused a transient fall in blood pressure In the perfusion of the dog's hind leg with heparinized blood, paludrine was shown to have a vasodilator action, 2 mg injected into the perfusion cannula decreased the pressure and increased the outflow of blood This vasodilatation was diminished by neoantergan. The same effect was observed on the blood pressure of a cat

anaesthetized with chloralose Both paludrine (4 mg) and histamine (10 μ g) caused a fall in blood pressure this fall was reduced by the injection of benadryl Thus the antihistamine agents, neoantergan and benadryl, reduced the vasodilatation caused by paludrine

Paludrine antagonized the action of adrenaline on both the dog's perfused hind leg and the cat's blood pressure The latter effect is shown in Fig 1 In both preparations the vasoconstruction produced by adrenaline was reduced by paludrine

Action on heart muscle—The refractory period of the auricle was lengthened by paludrine The compound was compared with quinidine by Dawes's method on the electrically stimulated rabbit auricles (Dawes, 1946), and it was found to be approximately one-eighth as active as quinidine (Table II)

On the isolated perfused cat heart (Langendorff preparation), paludrine (10-500 μ g) inhibited the amplitude and rate of beat according to the dose

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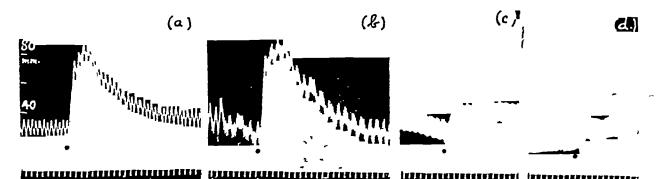


Fig 1—Blood pressure of chloralose cat from carotid artery (a) Adrenaline (5 μ g), (b) adrenaline (5 μ g) Between (b) and (c) paludrine (80 mg.) was infused intravenously (c) Adrenaline (5 μ g), (d) adrenaline (10 µg.) Time 10 sec

TABLE II

PERCENTAGE LENGTHENING OF THE REFRACTORY
PERIOD OF ISOLATED RABBIT AURICLES

The figures are averages of those from five experiments

Pa	ludrine	Quinidine			
Dose, mg	% Increase of refractory period	Dose, mg	% Increase of refractory period		
1 2 4	4 9 14	0 25 0 5 1 0	9 16 23		

The inhibition was accompanied by coronary dilatation

Paludrine depressed both the contractility and the rate of beat of isolated rabbit auricles. At the same time the normal inhibitory action of acetylcholine was changed to a stimulation. If paludrine was allowed to act for a longer time the auricles stopped beating acetylcholine would then restart the contractions. This action is described in more detail by Burn and Vane (1949). The isolated frog heart was also depressed by paludrine.

Action on striated muscle

On the isolated rectus abdominis of the frog paludrine had three distinct actions. When contractions of the muscle were obtained with acetylcholine, paludrine in very low concentration (50×10^{-7}) was found to augment these contractions. In concentrations higher than 10^{-6} , paludrine inhibited the action of acetylcholine. Finally, in concentrations of over 10^{-4} , paludrine alone caused the muscle to contract, the response to the same concentration of paludrine increased with

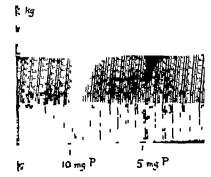


Fig 2—Cat sciatic-gastrocnemius preparation Contractions of muscle evoked by maximal stimulation of the sciatic nerve by square waves at 24 shocks per min Effect of two doses of paludrine injected into, the artery

successive applications Thus, paludrine had a biphasic action on contractions evoked by acetylcholine, in small concentrations it augmented the contractions, in higher concentrations it depressed them

Cat sciatic-gastrocnemius preparation — The muscle twitch evoked by single maximal nerve volleys was depressed by paludrine (2-20 mg) injected into the arterial blood stream (Fig 2) This curariform action was also observed on the isolated phrenic nerve-diaphragm preparation of the rat

Action on smooth muscle

Intestinal movements (in situ) - The intestinal movements of a cat, anaesthetized with chloralose, were recorded by a balloon tied into the Alterations in volume of the balloon were recorded by a water manometer and piston recorder A typical record is shown in Fig 3, the upper tracing is the record of the intestinal movements, the lower one that of the blood pressure The natural movements of the duodenum in this experiment were quite vigorous (a) venous infusion of paludrine (13 mg/min) lowered the tone of the muscle and abolished the The paludrine infusion was natural movements stopped and the natural movements of the gut slowly returned, (f) shows the effect of a single injection of paludrine, the intestinal movements were stopped almost immediately This effect was confirmed in three similar experiments The blood pressure record in this experiment showed an interesting effect, the heart was irregular and often dropped beats (see a and f) The slow infusion of paludrine eliminated most of these, and after the single injection of paludrine all the irregularities disappeared

Intestinal movements after vagal stimulation—In some cats prepared for the above experiment the natural movements of the gut were small. In these animals the right vagus nerve was exposed in the neck, cut, and the peripheral end stimulated by an induction coil for 30 sec, this produced a burst of motility in the duodenum. An intravenous infusion of paludrine decreased the response of the intestine to vagal stimulation, the activity recovered when the infusion was stopped.

Isolated intestine—The contractions of isolated guinea-pig ileum elicited by acetylcholine were inhibited by paludrine, and so were the contractions elicited by histamine. The inhibitions of the contractions were of the same order in each case Paludrine, in a concentration of 2×10^{-6} , decreased the natural tone of isolated rabbit duodenum, at

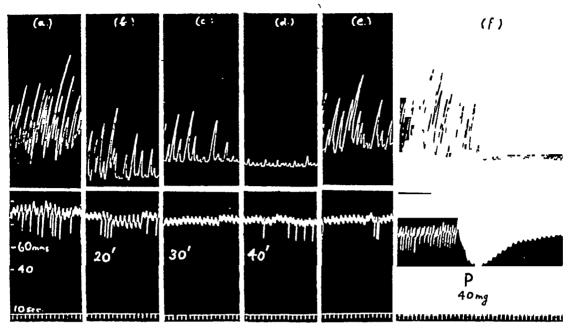


Fig 3 --Chloralose cat Top intestinal movements, recorded by a balloon in the duodenum Bottom blood pressure from carotid artery (a) Natural movements, infusion of paludrine started at 1 3 mg/min, (b) 20 min after start of infusion, (c) 30 min, (d) 40 min Infusion stopped (52 mg infused) Activity began to return, (e) 10 min later Eventually normal activity returned, as shown in (f) (1 hour later) A single injection of paludrine (40 mg) again stopped the movements Note effect on blood pressure and dropped beats Time 10 sec

the same time it augmented the response of the muscle to adrenaline

Splenic volume — The volume of the spleen of the cat was recorded by means of a plethysmograph attached to a piston recorder Paludrine injected intravenously had little effect on the blood pressure, but dilated the spleen. This was confirmed in six other experiments

Action on bronchioles—The bronchial tone was recorded by the method described by Konzett and Roessler (1940), and Emmelin, Kahlson, and Wicksell (1941), using the recorder described by Halpern (1942)

The guinea-pig was anaesthetized with urethane, and cannulae were inserted into the left jugular vein and the trachea. The lungs were artificially respired by a pump, the excess air was measured by the apparatus referred to above, so that increased excursion of the recorder on the smoked paper indicated broncho-constriction

Injections of paludrine (1–20 mg) into the jugular vein had in themselves no effect, but greatly enhanced the broncho-constriction caused by histamine Fig 4 shows this effect. Histamine (2 μ g) produced regular constrictions of the bronchioles the injection of paludrine (10 mg) increased the histamine effect (a) Paludrine (20 mg), whilst having no effect by itself, increased the histamine constriction so much that the guineapig died (b)

Respiration

Rabbits anaesthetized with urethane were used Respiration was recorded by Gaddum's method (1941) Paludrine (8-20 mg) reduced the respiration, but the depression did not last as long as the fall in blood pressure which accompanied it

In further experiments a section of the right vagus nerve was exposed in the neck. The nerve was cut and the central stump was stimulated by an induction coil for 5 sec once every minute This produced a constant inhibition of the respiration as shown in Fig 5a Paludrine (8 mg) injected intravenously caused a transitory depression of the respiration, after which the vagal effect was abolished The stimulation once every minute was continued and the vagal depression gradually returned to its normal value in about 20 min. In another experiment in which paludrine (4 mg) was injected, the effect reached its maximum at about 10 min after the injection In both these rabbits. as in four other experiments which gave similar results, the blood pressure remained constant, except for a small transient fall on injection of the paludrine

Ganglionic transmission

The superior cervical ganglion of the cat was perfused with Ringer-Locke solution by the method of Kibjakow (1933) Stimulation of the

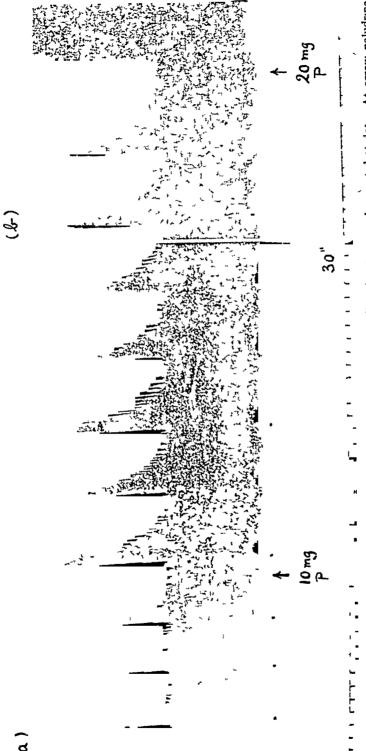


Fig. 4—(a) Record of constrictions of guinea-pig bronchioles induced by histamine (2 µg) intravenously injected at dot—At arrow paludrine (10 mg) was injected—It had no action by itself, but potentiated the histamine response—(b) Constrictions caused by histamine (3 µg)—Atarrow paludrine (20 mg) was injected—This had no effect, but the next histamine injection killed the animal—Time 30 sec



Fig 5—Respiration of rabbit recorded by Gaddum's method (a)
Central end of cut vagus nerve stimulated for 5 sec once every
minute (b) Paludrine (8 mg) injected at arrow. This caused
transient depression of respiration. Effect of vagal stimulation
abolished (c) Effect of stimulation gradually returning, the first
stimulation being 11 min after injection of paludrine. Time
30 sec

preganglionic fibres produced a contraction of the nictitating membrane this was recorded on smoked paper by a lever with a frontal writing point. Injection of paludrine into the infusion cannula depressed the response of the nictitating membrane (Fig. 6), this appeared with doses of paludrine exceeding 200 μ g

Action on secretions

The action of paludrine on salivary secretion in cats was compared with that of atropine by the method of Bulbring and Dawes (1945) Although paludrine (4–16 mg) gave a very transient inhibition, the inhibition was never as large as that produced by atropine (2 μ g)

The antidiuresis evoked by pituitary (posterior lobe) extract in rats was slightly prolonged by paludrine (10 mg/kg) when injected with the pituitary extract, the method described by Burn (1931) was used

Paludrine was found to inhibit the histamine-induced gastric secretion in cats, and also the gastric secretion evoked by a test meal in man. This effect is described in other papers (Burn and Vane, 1948, Vane, Walker, and Wynn Parry, 1948)

DISCUSSION

Late deaths after the intravenous injection of paludrine have been observed in rats and mice but not in chicks (Butler, Davey, and Spinks, 1947) It is interesting to note that late deaths also occurred after the oral administration of pamaquin, certuna, and mepacrine to fowls, and that the nervous symptoms which accompanied the delayed deaths prevented ingestion of food, the deaths sometimes being

hastened by starvation (Kohlschütter, Zipf, and Triller, 1943) Paludrine given to rats, mice, dogs, and monkeys in sublethal doses also diminished the intake of food, as described by Hughes, Schmidt, and Smith (1947b) Thus, delayed deaths and loss of appetite seem to be common properties of the synthetic antimalarial drugs pamaquin, certuna, mepacrine, and paludrine

Late deaths might also be due to interference with some enzyme system. Blaschko, Chou, and Wajda (1947) found that certain cholinesterases were inhibited by paludrine; the experimental results described in this paper show that when prostigmine was injected simultaneously with paludrine into mice the immediate deaths were increased and the delayed deaths decreased. This would be expected if the deaths were due to inhibition of the esterase by paludrine.

On the other hand, in most of its actions paludrine was found to antagonize the effect of vagal stimulation or of acetylcholine. The action of

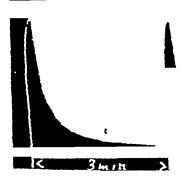




Fig 6—Perfusion of superior cervical ganglion Stimulation of preganglionic fibres produced contractions of the nictitating membrane shown in tracing Paludrine (0.5 mg.) injected into the perfusion fluid partially blocked the transmission

acetylcholine was inhibited on the cat intestine in situ, on the isolated guinea-pig ileum, on the isolated rabbit auricles, and on the isolated frog rectus muscle. In smooth muscle the natural tonus was reduced. Quinine, quinidine, cinchonine, pamaquin, and mepacrine were found by Keogh and Shaw (1943, 1944) first to stimulate, and then relax, isolated rat's intestine. The same authors also showed that the depressor response of the cat's blood pressure to acetylcholine was reduced by quinine. Hiatt, Brown, Quinin, and Macduffie (1945) found that quinine, quinidine, and cinchonine all inhibited the action of the vagus on the heart.

Dawes (1946) discussed the relationship between the inhibitory action of drugs on the acetylcholine response and their effect in prolonging the refractory period of the heart Paludrine has been found to lengthen the refractory period and to impair the contractility of isolated rabbit auricles Here, too, the other antimalarial drugs have a similar effect. Quinine, mepacrine, and pamaquin decreased the heart rate and lengthened the conduction time as shown by the ecg in man and in dogs (Molitor, 1941) The lengthening of the refractory period by quinidine, and to a lesser extent by quinine, is well known. Smith and Stockle (1946) showed that mepacrine impaired the contractility of the heart

A curariform action of paludrine has been demonstrated on the perfused superior cervical ganglion, on the cat sciatic-gastrocnemius preparation, and on the rat phrenic nerve-diaphragm preparation Harvey (1939) described a curariform action of quinine on the neuromuscular junction

Paludrine in large doses has been shown to antagonize the vasoconstriction caused by adrenaline in the dog's perfused hind leg and on the cat's blood pressure Keogh and Shaw (1943, 1944) found that quinine in large doses reversed the action of adrenaline on the cat's blood pressure

Molitor (1941) showed that mepacrine and pamaquin produced vasodilatation, as does quinine. In this work it has been shown that paludrine also produces vasodilatation in the dog's perfused hind leg and in the cat. The dilatation was partially abolished by the antihistamine agents, neoantergan and benadryl. This, with the work of MacIntosh and Paton (1947), who found that certain biguanides and amidines released histamine from muscle, suggested that paludrine may also release histamine from the tissues. The relationship between paludrine and histamine is very puzzling whereas paludrine inhibited gastric secretion evoked by histamine and reduced the

response of isolated guinea-pig ileum to histamine, it potentiated the action of histamine on the guinea-pig lungs. On the other hand the anti-histamine agents, benadryl and neoantergan, which abolish the effect of histamine in most tissues but potentiate the gastric secretion caused by histamine (Wood, 1948), reduced the action of paludrine on the systemic vessels

SUMMARY

Certain pharmacological actions of paludrine are described

- 1 In most experiments, paludrine antagonized the action of acetylcholine or of vagal stimulation contractions of the isolated frog rectus muscle and guinea-pig ileum were inhibited by paludrine and the normal action of acetylcholine on isolated rabbit auricles was abolished. The effects of vagal stimulation on the cat intestine and rabbit respiration were also reduced, as was the natural tonus of the intestine.
- 2 As with other drugs which antagonize acetylcholine, paludrine lengthened the refractory period of auricular tissue. It also had a curariform action on the cat sciatic-gastrocnemius, the rat phrenic nerve-diaphragm, and the perfused superior cervical ganglion preparations.
- 3 Paludrine reduced or abolished the vasoconstrictor action of adrenaline in the dog's perfused hind leg and on the cat blood pressure
- 4 Paludrine caused vasodilatation of the perfused dog hind leg and in the cat. This dilatation was reduced by injection of antihistamine agents, which suggested that paludrine might release histamine from the tissues. If this is so, then the relationship between histamine and paludrine is difficult to understand, for, whereas paludrine potentiates the constrictor effect of histamine on the guinea-pig lungs, it inhibits histamine-induced gastric secretion in cats. It also inhibits contractions of isolated guinea-pig ileum evoked by histamine.
- 5 The delayed toxicity observed in mice after the intravenous injection of paludrine was changed by the simultaneous injection of prostigmine, which increased the proportion of immediate deaths

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THE ACTION OF SYNTHETIC CURARIZING COMPOUNDS ON SKELETAL MUSCLE AND SYMPATHETIC GANGLIA BOTH NORMAL AND DENERVATED

BY

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A recently synthesized substance F2559 with curarizing properties was shown by Bovet, Depierre. and de Lestrange (1947) to differ from d-tubocurarine in its effect on the blood pressure. An intravenous dose of tubocurarine chloride which caused muscular paralysis in the dog lasting several hours also caused a fall in general blood pressure, but a dose of the synthetic compound with the same curarizing action did not depress the blood The fall of blood pressure after an injection of tubocurarine may be caused in two Firstly, it may be due to the release of histamine which was first observed by Anrep and his co-workers (Alam et al, 1939) However, Grob, Lilienthal, and Harvey (1947) showed that in man tubocurarine produced vascular changes only after intra-arterial injection and not when given intravenously The second mechanism by which tubocurarine may lower the blood pressure is by its action on sympathetic ganglia. When a comparison was made of the activity of the mono-,

F2512 O CH₃CH₅N(C₂H₆)₅I

β-diethylaminoethoxybenzene ethiodide

F2557 O CH₂CH₂N(C₂H₆)₃I

O CH₂CH₂N(C₂H₆)₃I

1 3-bis-(β-diethylaminoethoxy)benzene diethiodide

F2559 O CH₂CH₂N(C₂H₅)₃I

O CH₂CH₂N(C₂H₅)₃I

O CH₂CH₂N(C₂H₅)₃I

1 2 3-tri-(β-diethylaminoethoxy)benzene triethiodide

di-, and tri-quaternary ammonium salts shown below, of which the substance mentioned above is a member, it was found that the compound with the strongest action on the blood pressure had the weakest curarizing action on skeletal muscle, whereas the compound with the strongest action on the muscle had the weakest action on the blood pressure Moreover, Depierre (1947) showed that the ratio of doses of these compounds for blocking transmission in the superior cervical ganglion was similar to that for lowering blood pressure It appeared interesting to make a more detailed investigation of the action of these three compounds on the normal and denervated sympathetic ganglion and to compare it with their action on normal and denervated skeletal muscle

EXPERIMENTAL RESULTS

(1) Effect on the response of the nictitating membrane to preganglionic sympathetic nerve stimulation in the perfused superior cervical ganglion

Method —Cats under chloralose were used The ganglion was perfused with Locke's solution according to Kibjakow's (1933) method, modified by Feldberg and Vartainen (1934) The preganglionic nerve was stimulated maximally at a rate of 16 per sec for 15 sec every 4 min The compounds were injected into the arterial cannula one minute before each stimulation

Results—Depierre's finding was confirmed that F2559 was very weak in depressing synaptic transmission, the lowest dose having some effect being 1 mg in one experiment whereas in another as much as 10 mg was required. On the other hand, F2512 was found to be nearly as strong as d-tubocurarine chloride, acting in doses from 40 to 200 µg. Doses of F2557 causing synaptic depression were intermediate between those of the two other compounds. In six perfusions the average doses depressing synaptic transmission were 5 mg

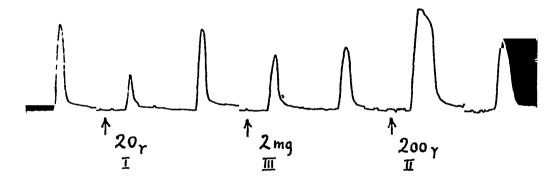


FIG 1—Cat, chloralose Perfused superior cervical ganglion Record of contractions of the nictitating membrane to intra-arterial doses of 50 µg acetylcholine I=F2512, III = F2559, II = F2557.

F2559, 500 μ g F2557, and 100 μ g F2512 If these doses are compared with those injected intraarterially to depress neuromuscular transmission (Section (4)) it will be seen that they are of similar magnitude but in the reverse order, the ratio being 50 5 1

(2) Effect on the contractions of the nictitating membrane induced by the injection of acetylcholine into the perfused superior cervical ganglion

Method — The method was the same as that in Section (1)

Results—Fig 1 shows the effect of the three compounds given in succession. A dose as small as 20 μ g F2512 reduced the response to acetylcholine by 50 per cent, it recovered very quickly As much as 2 mg F2559 were required to reduce the response by about 25 per cent, but recovery was slow. When now 200 μ g F2557 were injected the response was greatly augmented. About 400–500 μ g F2557 produced depression from which the ganglion did not recover. The ratio of paralysing doses was thus 100 20–1

The injection of the three compounds had no action by itself except that F2512, in a dose of 50-100 μg , sometimes caused a contraction of the nictitating membrane just as d-tubocurarine occasionally does

The surprising observation in these experiments was the sensitizing action of F2557 on the ganglion to the injection of acetylcholine Probably this action is related to the inhibition of cholinesterase by this compound which will be discussed below

- (3) Effect on the contractions of the nictitating membrane induced by the injection of acetylcholine into the denervated ganglion
 - (a) Administration of doses into the lingual artery

Method —Seven to eight days after the cervical sympathetic nerve had been cut the cats were

anaesthetized with chloralose A loop was put round the external carotid artery, which was temporarily occluded while injections were made through a cannula inserted into the lingual artery and pointing backwards to the common carotid

Results -A slight peripheral action on the nictitating membrane itself, producing a slow small contraction, was observed after the injection of acetylcholine even after removal of the ganglion at the end of the experiment However, the contractions of the membrane produced by the ganglionic action of acetylcholine were immediate and much larger, and the following results were No dose of F2559 up to 10 mg obtained depressed the response to acetylcholine of F2557 up to 5 mg caused sensitization like that seen in Fig 1, larger doses, up to 10 mg, caused some depression. The most active substance of the three was F2512, which depressed the acetylcholine response in a dose of about 100 ug, in this dose it was once observed to cause a contraction by itself The ratio of paralysing doses was thus $> 100 \ 100 \ 1$

(b) Experiments on the perfused denervated superior cervical ganglion

Method—This was the same as in (1)

Results —Again it was found that F2559 in doses up to 10 mg had no action, 5 mg F2557 caused sensitization and 10 mg depression, 100 μ g F2512 caused a big contraction by itself (as 40 μ g tubocurarine chloride did in this same experiment) and depressed the response to acetylcholine by 50 per cent The ratio of paralysing doses was again > 100 100 1

Thus the difference between the responses of normal and denervated ganglia to acetylcholine was that about five times the amount of the curarizing agent was required to depress the action of acetylcholine in denervated ganglia. The activities

of the three substances remained in the same order but the ratios were very much greater

(4) Effect on the response of skeletal muscle to stimulation of the motor nerve

Method—Cats anaesthetized with chloralose were used The contractions of the gastrocnemius to maximal single shocks (15 per min) applied to the sciatic nerve were recorded. The compounds were injected intra-arterially through a cannula inserted into the opposite iliac artery and pointing towards the bifurcation of the aorta.

Results — The ratio of intra-arterial doses required to reduce muscular contractions to

about 50 per cent was found to be the same as that of intravenous doses. The curarizing intravenous doses were 500-750 μ g of F2559, 1-2 mg of F2557, and 20 mg of F2512 per kg respectively (Depierre, 1947). By the arterial route they were 200 μ g, 400 μ g, and 4 mg per kg. The ratio of curarizing doses was thus 1 2-4 20-40

(5) Effect on the response of skeletal muscle to close arterial injections of acetylcholine

Method—The tibialis anterior muscle preparation was used as described by Brown (1938) and acetylcholine was injected into the tibial artery. The compounds, however, were injected intra-

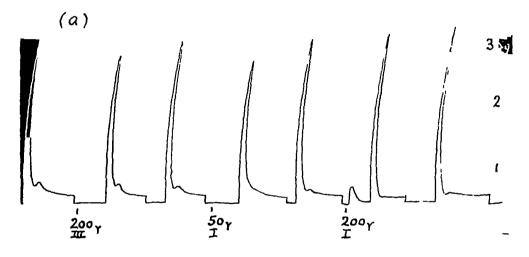




Fig 2—Cat, chloralose Record of contractions of denervated gastrochemius evoked by intra-arterial injections of acetylcholine (a) Exp No 12, muscle denervated for 99 days dose of acetylcholine = 30 µg (b) Exp No 2, muscle denervated for 9 days dose of acetylcholine = 100 µg I = F2512, III = F2559 T = d-tubocuranne chloride Note that I and T depress the response in small doses and augment it in large doses

venously because it was found that the muscle did not recover if they were given by close arterial injection

Results—The doses required to produce a depression of the muscle contraction of 20 per cent were 0.4 mg F2559, 1.5 mg F2557, and 5 mg F2512 For a depression of 75 per cent the doses required were 0.75 mg, 2 mg, and 100 mg respectively. Thus the ratio was found to be about 1.4.12

(6) Effect on the response of denervated muscle to the distant arterial injection of acetylcholine

Method — The sciatic nerve was cut 6-30 days before the experiment The cats were anaesthetized

with chloralose The contractions of the gastrocnemius were recorded, and the injections of acetylcholine, as well as of the curarizing compounds, were made into the iliac artery as in (4)

Results—The action of the three compounds on denervated muscle was found to be very complex F2559 was not so potent as F2512 when given in small doses, but as the dose of F2559 was increased the paralysing effect on the muscle response became greater. On the other hand, F2512 depressed the response of denervated muscle to acetylcholine in small doses only, as the dose of F2512 was increased its paralysing effect became less and less, and when given in large doses it actually increased the response to acetylcholine (Fig. 2a) F2557 was found to depress the muscle response to

TABLE I

PERCENTAGE CHANGES IN THE RESPONSE OF DENERVATED MUSCLE TO ACETYLCHOLINE PRODUCED BY DIFFERENT DOSES OF F2559 AND F2512

F2559

					1 43.	77				
No of exp	Dose of ACh µg	20 μg	_ 50 μg	100 μg	200 μg	400– 500 μg	1 mg	2 mg	4 mg	Days denervated
2 3 4 5 6 8 8 10 11 12	100 20 -100 50 20 35 50 20 50 30		-16 -10 +8 0 +10	-20 +2 +5	-40 -10 -20	-17 +5 0 -62 -16 -14	-50 -12 -44 -19	-10	+30	9 11 11 14 14 20 20 20 20 21
Mean			-2	-4	-20	-17	-31	-10	+30	

					F2512				
No of exp	Dose of ACh µg	20 μg	40– 50 μg	80~ 100 μg	150– 200 µg	400- 500 μg	600– 800 μg	1- 1 2 mg	Days denervated
1 2 4 5 6 7 8 8 9 10 11	20 100 100 50 20 30 35 50 50 20 50	-13 62	-31 -35 -11 0 -13	+16 33 0 -12 0	-40 0 0 -11 -4 +7	-14 -10 +29 -28 +2 +5	+22 5	+4 +20 -8 +18 +10	6 9 11 14 14 15 20 20 20 20 21 29
Mean		-37	-18	· -6	-8	-4	+8	+9	

acetylcholine in relatively large doses only (1–2 mg) No augmentation of the muscle response was observed (though this was regularly seen in the sympathetic ganglion), but as this compound possesses some anticholinesterase activity (see Section (8)), which would naturally interfere with its action, it was not examined so thoroughly as the other two compounds

The results of 12 experiments in which the effects of several doses of F2559 and F2512 were compared are given in Table I In order to make such a comparison doses of acetylcholine were given at intervals of 15 min. One minute before the injection of acetylcholine the curarizing compound was given, and care was taken not to cause too large a depression, as otherwise the previous muscle response to acetylcholine would not return, for this reason the percentage changes of the muscle response are mostly small, but in each experiment it will be seen that the depressing action of F2559 increased with increasing doses, whereas that of

F2512 diminished when larger doses were used The latter compound had a stimulating action itself, in doses of 100 μ g or more it caused a muscle contraction, and it was in this range, from 0.1 to 1.0 mg, that it often did not diminish but increased the subsequent response to acetylcholine F2559 in doses of about 1 mg caused a considerable depression. In one experiment only had F2559 a stimulant action itself, 1 mg caused a small muscle contraction (see Fig. 3 (a)) and depressed the subsequent response to acetylcholine, whereas 4 mg caused a large contraction and sensitized the muscle to a subsequent injection of acetylcholine (Exp. 6 in Table I)

If the percentage changes shown in Table I are taken as an index of the activities of the two compounds it will be seen that F2512 appears to have a much stronger paralysing action than F2559 in the dosage range 20–50 μg , whereas in the range 200–500 μg this ratio of potency is completely reversed

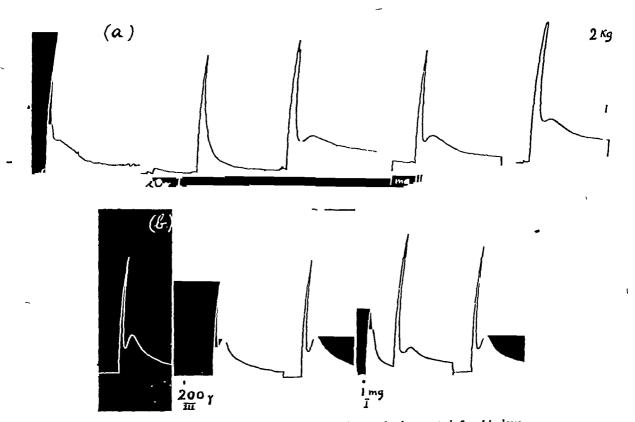


Fig 3—Records as in Fig 2 (a) Exp No 6, muscle denervated for 14 days, dose of acetylcholine = $20 \,\mu g$ (b) Exp No 5, muscle denervated for 14 days, dose of acetylcholine = $50 \,\mu g$ I = F2512, III = F2559 Note that $20 \,\mu g$ I depress, 1 mg I augments quick phase, both depress slow phase, while III depresses quick phase only

The difficulties encountered in assessing the relative activities of the two compounds may be seen in Fig 2 (a) In this experiment 200 µg F2559 and 50 µg F2512 were found to be equiactive doses However, when the dose of F2512 was increased to 200 µg it produced not more depression but instead an augmentation of the muscle response to acetylcholine This sensitizing action was still seen in the response to acetylcholine 15 min later A similar effect obtained with d-tubocurarine chloride is shown in Fig 2 (b) A small dose, 1 e, 10 µg, caused some depression (as 50 µg F2512 in (a)), but a larger dose, 100 μ g, not only caused a muscle contraction by itself (as 200 µg F2512 in (a) but increased the three subsequent responses to acetylcholine It may also be noted that whereas the first quick phase of the contraction was increased the second slow phase was Again, F2512 usually, though not always, depressed the slow phase more than the quick phase, whereas F2559 depressed the quick phase and usually left the second phase unchanged An example of this is given in Fig 3

The sensitizing action of F2512 was clearly related to its own stimulating action, as it mostly occurred after the compound itself had elicited a muscle contraction, thus, in comparing its paralysing activity with that of F2559 the results obtained with small doses are the more important ones. In the experiments Nos 1, 8, and 9 it was possible to observe a progressive depression with increasing doses and then in Nos 1 and 9 a change to augmentation. In the remaining experiments the threshold was obviously below the smallest dose employed.

Table I also shows that the action of the two compounds was independent both of the number of days the muscle had been denervated and of the dose of acetylcholine used to elicit the muscle contraction

(7) Effect on the response of the isolated diaphragm to stimulation of the phrenic nerve

Method—The preparation was that described by Bulbring (1946) and the test was carried out as described by Chou (1947) Rats were used as well as a newborn kitten

Results—The ratio of potency of the three compounds on the rat's isolated muscle was different from that on the kitten's muscle, which was, however, found to be similar to that on the cat's muscle in situ. This species difference was first pointed out to us in a personal communication from Dr J W Trevan and has recently been reported by Wien (1948) On the rat's diaphragm

a 50 per cent depression of maximal muscle contractions was produced by 6-8 mg F2559, 15-22 mg F2557, and 3-7 mg F2512 in a 50 cc bath, which gives a ratio of 1 2 05-1, thus on the rat's muscle F2559 and F2512 were of about equal strength, but on the kitten's muscle F2559 was about 10 times stronger than F2512, which is a similar result to those obtained on the cat's muscle in situ

(8) Effect on cholinesterase

Method — The details of the method used are described by Bülbring and Chou (1947) Only the inhibition of "true" cholinesterase (dog's caudate nucleus suspension) has been studied Mrs P Holton very kindly carried out these experiments for us

Results—It was found that F2559 and F2512 had no anti-cholinesterase activity in a concentration of $10^{-3}M$ F2557 was found to have a weak inhibitory action. The results are given in Table II. The concentration of acetylcholine used in these experiments was $6 \times 10^{-3}M$

TABLE II
INHIBITION OF CHOLINESTERASE (DOG'S CAUDATE NUCLEUS)
BY F2557

E		Percentage 1	nhibition	at
Experiment	10-4M	$3 \times 10^{-4}M$	10 ³ M	$3 \times 10^{-8}M$
1	10 -		50	_
3	7 15	26	47 41	6 4

DISCUSSION

The compounds investigated are of special interest because they show a remarkable discrepancy between their paralysing action on neuro-muscular transmission and their blocking action on synaptic transmission in the sympathetic ganglion. From the doses required to cause a given depression of the response Table III has been constructed, which gives the relative activities of the three compounds on various preparations.

The ratio of activity on denervated muscle could not be assessed, since it varied with the dose employed. Thus, if small doses of the curarizing substance, e.g., 20–100 μ g, were used, F2512 was found to be 10–20 times stronger than F2559. When larger doses were given, e.g., 200 μ g –500 μ g, the ratio was reversed and F2559 was found to be 2–5 times stronger than F2512. When still larger doses were used the stimulant action of F2512 itself and its sensitizing effect on the

TABLE III

Preparation	Relative activities			
	F2512	F2557	F2559	
Sympathetic ganglion stimulated through preganglionic nerve	100	20	2	
Sympathetic ganglion stimu- lated by intra-arterial acetyl- choline Denervated sympathetic gang-	100	5	1	
lion stimulated by 1 a acetyl- choline	100	1	1	
Skeletal muscle stimulated through motor nerve	2-5	25–50	100	
Skeletal muscle stimulated by intra-arterial acetylcholine	8	25	100	

response to acetylcholine became prominent and the comparison of the two compounds became *impossible* F2557, which depressed the muscle response to acetylcholine only in large doses of about 1 mg, was again intermediate in potency between the two other compounds However, this substance has some anti-cholinesterase activity and this was probably responsible for the augmentation of the action of acetylcholine in the sympathetic On the muscle we never observed this ganglion augmentor action, but we did not include F2557 - in the detailed comparison on denervated muscle

A striking similarity was found to exist between the inhibitory effect of F2512 and F2559 on the action of acetylcholine in the normal sympathetic ganglion and in the denervated skeletal muscle This was evident in a certain range of doses, in which the stimulant action of the compounds themselves was too small to interfere normal sympathetic ganglion the potency of F2512 was found by Depierre (1947) to be very similar to that of d-tubocurarine, though the effect of F2512 is short-lasting. In denervated muscle we observed the same similarity. Not only were the doses of the two substances required to depress the action of acetylcholine of the same magnitude, but d-tubocurarine was also found to have a stimulant action itself and to sensitize the denervated muscle to subsequent injections of acetylcholine This observation supplements that of McIntyre and King (1943), who found that in the dog stimulant doses of d-tubocurarine rendered the muscle unresponsive to previously effective quantities of acetylcholine It may be that the doses of d-tubocurarine used by McIntyre were larger than those We found that stimulant doses of we employed both substances do not necessarily depress the subsequent muscle response to acetylcholine

Both d-tubocurarine and F2512 depress in small doses, with larger doses they sensitize, and with still larger doses they depress once more

SUMMARY

- 1 The action of β -diethylaminoethoxybenzene ethiodide (F2512), 1 3-bis- $(\beta$ -diethylaminoethoxy)benzene diethiodide (F2557), and 1 2 3 - tri - (β diethylaminoethoxy)benzene triethiodide (F2559) was studied on skeletal muscle and the sympathetic ganglion, both normal and denervated
- 2 On normal skeletal muscle F2559 has a strong curarizing action both when the muscle is stimulated through its nerve and when it is exposed to intra-arterial injections of acetylcholine, F2512 is This ratio of activity is 12 to 50 times weaker reversed on the sympathetic ganglion whether it is stimulated through its preganglionic nerve or by intra-arterial injection of acetylcholine, F2512 being 50 to 100 times stronger than F2559
- 3 On denervated skeletal muscle, sumulated by intra-arterial injection of acetylcholine, the ratio of activities of F2512 and F2559 is the reverse of that in normal muscle, provided that the doses of the curarizing agents are small F2512 is then, as in the normal sympathetic ganglion, 10-20 times stronger than F2559
- 4 Large doses of F2512 have a sensitizing action on denervated muscle, as the dose is increased the diminution of the muscle response to acetýlcholine gives way to an augmentation This effect has also heen observed with d-tubocurarine
- 5 F2512 and d-tubocurarine both have a stimu lant action by themselves on the normal as well as on the denervated sympathetic ganglion and on the denervated muscle
- 6 F2557 causes a 50 per cent inhibition of "true" cholinesterase (dog's caudate nucleus suspension) in a concentration 10-3M F2512 and F2559 do not inhibit cholinesterase in this concentration

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TUBOCURARINE ANTAGONISM AND INHIBITION OF CHOLINESTERASES

BY

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In a recent paper Bulbring and Chou (1947) compared the activities of various substances with that of prostigmine as antagonists to tubocurarine and as inhibitors of cholinesterase. The results obtained with ethyl homologues of prostigmine suggested that a parallelism might exist between the two activities, and for this reason a larger series of chemical compounds has now been investigated

All the substances studied are given in Table I Compounds 3392 and 3393, the mono- and diethyl homologues of prostigmine, are the two substances previously examined, compound S208

TABLE I

Eserine sulphate

is the N-triethyl homologue of the same series Compound 5220/5 is an amine oxide closely related to prostigmine, this substance is of interest as a prostigmine derivative in which the quaternary ammonium group has lost its strongly basic character. Compounds Nu 1250 and Nu 1197 are known to be strong inhibitors of true cholinesterase (Aeschlimann and Stempel, 1946), and the compound Nu 683 was included because it is

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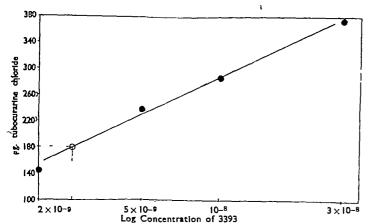


Fig 1—Evaluation of antitubocurarine activity on the rat's phrenic nerve-diaphragm Ordinate = dose of d-tubocurarine chloride in μg Abscissa = log concentration of 3393 The circle indicates the point from which the pD 20 value is found (see text)

known to be a specific inhibitor of pseudocholinesterase (Hawkins and Gunter, 1946)

METHODS

For the estimation of the anticurare activity the isolated phrenic nerve diaphragm preparation of the rat (Bülbring, 1946) was used The quantitative evaluation was based on the fact that the dose of tubocurarine chloride required to cause a certain reduction of muscle contractions in the presence of an antagonist is proportional to the concentration of the antagonist. Bülbring and Chou (1947) expressed the potency of any antagonist in terms of that of prostigmine, which they used as a standard comparison of anticurare activity with anticholinesterase activity it was, however, necessary to express the potency by an absolute rather than by a relative This was done as illustrated in Fig 1, in which the mean results of five experiments are shown Each point represents the relation between a given concentration of 3393 (abscissae) and the dose of d-tubocurarine chloride (ordinates) which caused 20 per cent depression of the muscle contractions A dose of d-tubocurarine chloride had to be chosen which was antagonized by most of the substances investigated within the range of concentrations used, this was 180 µg. The corresponding concentration of the antagonist was found by interpolation as shown in Fig 1 For four compounds (38, Nu 683, 5150,

and 5220/5) it had to be found by extrapolation. The potency of any tubocurarine antagonist was thus expressed as the negative logarithm of the concentration in the presence of which 180 µg d-tubocurarine chloride caused 20 per cent depression of the muscle contractions. This is the pD 20 value, the figures are given in Table III.

The method of estimating anticholinesterase activity was the same as that described by Bülbring and Chou (1947). The inhibitory action on both true and pseudo-cholinesterase was studied. Each substance was therefore incubated with dog's brain (caudate nucleus) and acetyl choline (for its action on true cholinesterase) and also with horse serum and benzoylcholine (for its action on pseudo cholinesterase). Table II gives the quantities of enzyme preparations and substrates used in our experiments.

RESULTS

For the estimation of anticholinesterase activity several different concentrations of the inhibitor were' tested An absolute value of inhibitory activity was obtained by finding an index which was called pI 50 Fig 2 shows all the results obtained with different concentrations of prostigmine on the two enzymes Each point corresponds to one observation, and the curves are drawn through the means for each prostigmine The abscissa of the point where concentration the curve crosses the 50 per cent line is the negative logarithm of the molar concentration which causes 50 per cent inhibition this is the pI 50 value It can be seen in Fig 2a that the pI 50 value obtained with dog's brain was 74 actual figures in eight individual experiments were 72, 76, 75, 71, 75, 73, 73 With horse serum the individual figures in four experiments (Fig 2b) were 72, 71, 735, 715, and the mean was thus 72 Koelle and Gilman (1946) used a similar Whenever possible index which they called pK the pI 50 value for each enzyme preparation was determined with the inhibitor to be tested and with prostigmine on the same day

TABLE II

Type of enzyme	Source and amount of enzyme preparation added to each flask (Total vol = 3 c c)	Substrate
True cholmesterase	4 mg dog's caudate nucleus	$6 \times 10^{-3} M$ acetylcholine
Pseudo-cholinesterase	2 c c horse serum	$6 \times 10^{-3}M$ benzoylcholme

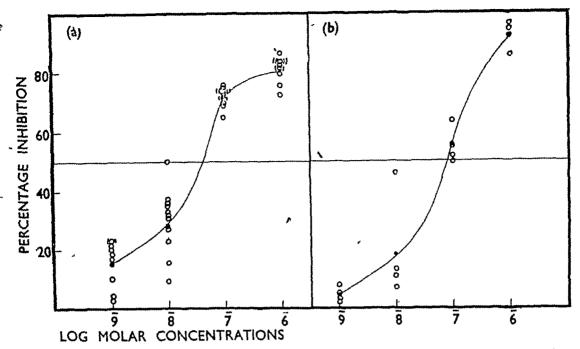


Fig 2—Evaluation of anticholinesterase activity of prostigmine (a) Dog's caudate nucleus, (b) horse serum of prostigmine of prostigmine of prostigmine of prostigmine the curves

Ordinates = percentages inhibition Abscissae = log molar concentration The pI 50 value is found from the point where the 50 per cent line crosses the curves

The ethyl komologues of prostigmine—Since the earlier publication of Bulbring and Chou (1947) the triethyl homologue of prostigmine (S208) has become available. It had been found previously that the substitution of one methyl group on the quaternary nitrogen by ethyl increased the activity both as inhibitor of the two cholinesterases and as antagonist to tubocurarine. The introduction of the second ethyl led to a still further increase in activity. We have now examined S208 and have found that the introduction

of the third ethyl group decreases the activity in both respects below that of prostigmine itself. The figures will be found in Table III, in which all the results are summarized

The amine-oxide homologue of prostigmine (5220/5)—This compound was without any anticurare activity in the concentration tested. The pD 20 value was determined by extrapolation and was the lowest of all. Its inhibitory action on both enzymes was also weak. It is known that the amine oxides of the general structure

TABLE III

Compounds	Mol	Anticurarine	Anticholinesterase activity pI 50 value		
Compounds	weight	activity pD 20 value	On true cholinesterase	On pseudo- cholinesterase	
Prostigmine (RNMe ₂)* 3392 (RNMe Et) 3393 (RNMeEt ₂) S208 (RNEt ₁) Miotine HCl Eserine sulphate No 38 Nu 1250 Nu 1197 Nu 683 5130 5220/5 (RNMe ₂ OH)	334 336 350 392 254 648 354 339 7 379 3 388 251 240 5	7 60 8 19 8 57 6 59 7 85 7 21 6 23 7 26 7 31 5 44 5 26 4 77	7 4 8 0 8 2 7 2 7 1 7 1 7 4 6 9 6 2 6 4 4 5	7 2 7 3 8 0 7 4 6 4 7 7 7 6 7 9 7 1 8 5 5 8 4 4	

R,NOH} OH (where R is an alkyl or aromatic radical) are bases with very small dissociation constants and in aqueous solution they exist almost entirely as undissociated molecules. For instance, the salts of trimethylamine oxide, Me,NOH}I, show an acid reaction in solution. Our results therefore reveal that the strongly basic character of the phenolic nitrogen radical is indispensable for a substance with strong anticholinesterase and anticurare activity.

Substances with predominantly inhibitor action on pseudo-cholinesterase -- Hawkins and Gunter (1946) found that the substance Nu 683 was a strong inhibitor of pseudo-cholinesterase, whereas it affected true cholinesterase only in very high concentrations In the living animal it produced symptoms of acetylcholine poisoning only in doses which significantly reduced the action of the true enzyme The authors concluded that these pseudo-cholmesterase results indicated that played no essential part in the hydrolysis of acetylcholine in vivo We found that both the anticurare action of Nu 683 and its inhibitory action on true cholinesterase were low, whereas its action on pseudo-cholinesterase was high

The parallelism between anticurarine activity and inhibitory action on true cholinesterase --Inspection of Table III will show that the different substances do not always have the same inhibitory action on both esterases, some inhibit pseudo-cholinesterase more strongly than true On the whole. cholinesterase and vice versa however, there is good agreement between the degree of inhibitory activity on true cholinesterase and that of antitubocurarine activity, whereas no such correlation exists between the action on pseudo-cholinesterase and tubocurarine antagon-We are very grateful to Dr W Perry for the statistical analysis of the results in Table III, by which it was established that a significant correlation exists between the values for anticurare activity and those for anti-true-cholinesterase activity (P<0 001), whereas there is no indication of a similar correlation between anticurare and anti-pseudo-cholinesterase activities (P > 0.1)

DISCUSSION

Our results show that there is a significant correlation between antitubocurarine activity and inhibitory action on true cholinesterase. This supports the view of Hawkins and Mendel (1947) that it is the true cholinesterase which is responsible for the destruction of acetylcholine at the site of its physiological action.

Aeschlimann and Stempel (1946) have estimated the inhibition of a purified specific cholinesterase preparation from the electric eel by Nu 1250 and Nu 1197 They found that the anticholinesterase activity of Nu 1250 was five times, and that of Nu 1197 more than equal to, that of prostigmine We find that the first substance has the same activity as prostigmine, and the second substance has less activity A possible reason for this discrepancy is the different source of the enzyme, we have used a suspension of the dog's caudate nucleus and consequently our tests both for anticurare activity and for anticholinesterase activity were carried out with mammalian tissues

It is known that structural changes of the prostigmine molecule sometimes increase the affinity for one enzyme and decrease it for the other One example is Nu 683, which is of special interest here as the decline in inhibitory action on true cholinesterase finds its parallel in weaker anticurare activity

An important result of our experiments is the finding that a strongly basic nitrogen radical in the phenolic moiety of the molecule is indispensable not only for a strong anticurare activity but also for a strong anticholinesterase activity

The ethyl homologues of prostigmine provide an interesting group of compounds substitution of the methyl groups on the quaternary nitrogen of prostigmine by ethyl at first increases both the antitubocurarine and the anticholinesterase activity, but the peak is reached at the diethyl compound and the activity of the triethyl compound is again much less

SUMMARY

1 The antitubocurarine activity of a series of compounds related to prostigmine was compared with their inhibitory action on the true cholinesterase of dog's caudate nucleus and on the pseudo-cholinesterase of horse serum

2 Significant correlation was found between the inhibition of true cholinesterase and the

antagonism to d-tubocurarine

Thanks are due to Dr J A Aeschlimann and Dr F Bergel for some of the substances used

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THE ACTION OF LOCAL ANAESTHETICS AND d-TUBOCURARINE ON THE ISOLATED INTESTINE OF THE RABBIT AND GUINEA-PIG

RY

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This paper describes the action of local anaesthetics and of d-tubocurarine chloride on isolated intestinal preparations In order to distinguish experimentally between the action of these substances on the muscle fibres and their action on the nervous structures present in the preparation the following procedure was adopted an effect on the tone and spontaneous rhythm of the muscle and on the responses to acetylcholine or histamine was taken as evidence for action on the excitability of the muscle fibres, on the other hand the nicotine response and the local peristaltic reflex provided reactions with which the excitability of the nervous structures in the intestine could be tested

METHODS

The experiments were carried out on isolated preparations of the rabbit's and guinea-pig's ileum suspended in aerated Tyrode's solution A 16 cc bath was used when the contractions of the longitudinal muscle alone were recorded, this was done with a Lovatt Evans frontal writing lever experiments on the peristaltic reflex the intestinal volume was recorded according to the method described by Trendelenburg (1917) recorder of the Brodie type was used for this purpose, and the bath volume was 35 cc The method of suspending the intestinal preparation was identical with that described by Feldberg and Solandt (1942) The temperature of the bath was kept at 37°C when the rabbit's, and at 35° C when the guinea-pig's, ileum was used The animals were killed by a blow on the head immediately before the intestine was removed

The local anaesthetics used were cocaine hydrochloride, procaine hydrochloride, and nupercaine hydrochloride. The amounts stated in the text refer to the salts and not to the bases

RESULTS

I Effect on the excitability of the muscle fibres

Movements and tone of the muscles of the small intestine of most animals are increased by small doses of cocaine and decreased by large ones (von Anrep, 1880, Bayliss and Starling, 1899, Langley and Magnus, 1905, Kuroda, 1915, and Trendelenburg, 1917) On the guinea-pig's intestine, however, the action of cocaine was found by Trendelenburg to be always depressant In our experience this was not so

Guinea-pig—In some preparations small doses of cocaine had a stimulating effect. The contractions of the longitudinal muscle were usually found to be small and transient and obtainable only when cocaine was added to the bath for the first time. In a few preparations, however, cocaine produced strong and well-maintained contractions which could be obtained with each new administration of the drug and resembled the contractions produced by acetylcholine or histamine. Even when cocaine had no stimulating action of its own, small doses often rendered the preparation more sensitive to the stimulating action of histamine or acetylcholine.

In order to obtain the depressant action of cocaine it had to be given in concentrations stronger than 1 in 80,000 (see Fig 1b). There might then be some relaxation of the tone of the longitudinal muscle and reduction of the spontaneous contractions if they were present. The most obvious effect, however, was the reduction in the response to acetylcholine, histamine, or potassium. The depression was greatest during the first few minutes of the cocaine action and decreased somewhat when the cocaine was kept in the bath for longer periods. After the cocaine had been

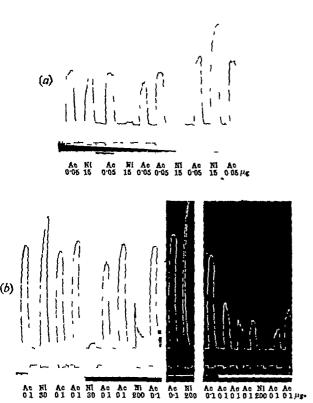


Fig 1—Contractions of guinea-pig's ileum in 16 c c
Tyrode's solution At (Ac) acetylcholine chloride,
at (Ni) nicotine tartrate in µg, as indicated at the
bottom of the figure, added to the bath. The broad
white lines indicate the periods during which the
bath contained cocaine, 1 in 80,000 (first two white
lines) and 1 in 25,000 (last white line)

washed out the muscle quickly regained its original sensitivity, in fact for 10 to 15 min it became even more sensitive to histamine or acetylcholine than it had been before the cocaine administration (see Fig. 1)

During a cocaine depression the muscle was unable to contract as strongly as the untreated muscle even if acetylcholine or histamine were given in hundredfold increased amounts. The extent of the reduction in contractibility depended on the concentration of cocaine present in the bath, becoming more pronounced with increasing concentration.

Other local anaesthetics examined depressed the muscle fibres in weaker concentrations than cocaine. A reduction of the acetylcholine response similar-to that seen with 1 in 35,000 cocaine was seen with concentrations of 1 in 600,000 procaine and 1 in 800,000 nupercaine.

d-Tubocurarine chloride, even if given in strong concentrations (1 in 80,000 to 1 in 16,000), did not affect the muscle tone and had only a slight and irregular effect on the responses to acetylcholine and to histamine. The responses to histamine were sometimes slightly depressed, sometimes slightly augmented, those to acetylcholine, however, were usually slightly depressed, the depression did not become more pronounced when the concentration of tubocurarine was increased. After a strong concentration of tubocurarine had been washed out there was often a period of a few minutes in which the response to acetylcholine was augmented

Rabbit —In some preparations cocaine increased, in others it decreased, the amplitude of the rhythmic contractions and caused slight loss of tone of the longitudinal muscle When different parts of the intestine were examined inhibition by cocaine was found to occur more regularly in the duodenum and jejunum and stimulation in the ileum, so that sometimes cocaine had opposite effects in different parts of the same intestine. In order to obtain inhibition of the acetylcholine response cocaine had to be given in concentrations higher than 1 in 30,000, except in preparations which had been kept suspended in the bath for several hours and had become more sensitive to the depressant action of cocaine Nupercaine was about 20 times as active as cocaine A sımılar inhibitory action of other local anaesthetics on the rabbit's duodenum has been described by Tripod (1940), and more recently by Dawes (1946) and de Elío (1948)

d-Tubocurarine chloride in a concentration of 1 in 30,000 or stronger usually caused slight and transient augmentation of the tone and amplitude of the rhythmic contractions. Since this effect was not prevented by the presence of a strong concentration of local anaesthetics in the bath fluid, it could not have been due to stimulation of the ganglion cells in the intestinal wall. In some preparations weak concentrations of tubocurarine (about 1 in 200,000) had a slight depressant effect on the response to acetylcholine, in others such an effect was obtained with very strong concentrations only (1 in 40,000 to 1 in 16,000)

II Effect on the nervous structures in the intestinal preparation

(a) The nicotine response of the guinea-pig's ileum. The addition of small doses of nicotine to the bath in which a guinea-pig's ileum is suspended produces contractions which differ from those produced by acetylcholine or histamine in that the

onset is delayed for a few seconds and the contraction itself proceeds more gradually. If a few minutes are allowed each time after the nicotine has been washed out and before its renewed administration, comparable results can be obtained without manifestations of a paralysing action of nicotine, but when such an experiment is continued for long periods the responses to nicotine often become gradually smaller

The nicotine contractions are abolished by concentrations of local anaesthetics or tubocurarine chloride which do not yet affect or affect slightly only equally strong contractions produced by histamine or acetylcholine In the experiment of Fig 1, doses of 15 and 30 μ g of nicotine had stronger actions than 005 and 01 µg of acetylcholine respectively Cocaine (1 in 80,000) prevented the nicotine responses but left those to acetylcholine The preparation was not practically unaffected completely insensitive to nicotine, which in a dose of 200 µg caused a small contraction. In order to abolish this response the concentration of cocaine had to be increased to such an extent that it reduced the response to acetylcholine, ie, the excitability of the muscle fibres (Fig 1b) experiment like that for cocaine in Fig 1a is shown for tubocurarine in Fig 2

In the experiment of Fig 3 the procedure was slightly modified. It illustrates that an acetylcholine, but not a nicotine, contraction is maintained in the presence of 1 in 80,000 cocaine, relaxation of the nicotine contraction started 13 sec after adding the cocaine to the bath

The inhibiting effect of cocaine and tubocurarine on the nicotine response is not so strong as that produced by paralysing doses of nicotine itself

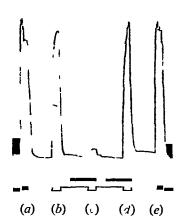


Fig 2—Contractions of guinea-pig's ileum in 16 c c Tyrode's solution At (a), (c), and (e) 40 μ g nicotine tartrate, at (b) and (d) 0 4 μ g acetylcholine chloride for 1 min The broad white lines indicate the presence of 200 μ g d-tubocurarine chloride in bath

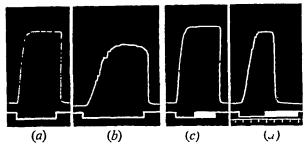


Fig 3—Contractions of guinea-pig's ileum in 16 c c
Tyrode s solution At (a) and (c) 0 4μg acetylcholine
chloride kept in the bath for 2½ min, but followed
after the 1st min at (c) by 200 μg cocaine At
(b) and (d) 35 μg nicotine tartrate kept in the bath
for 4 min, but followed after the 2nd min
at (d) by 200 μg cocaine Period during which
cocaine was present in bath indicated by broad
white line Time in 30 sec

In the presence of 1 in 200,000 nicotine the preparation is paralysed to any dose of nicotine, but 1 in 40,000 tubocurarine chloride renders the preparation insensitive to small doses only of nicotine, the effect of 100 or 200 μg of nicotine being greatly reduced but not abolished Usually the sensitivity of the preparations to the inhibitory action of cocaine and tubocurarine increased after a few hours' suspension in the bath of Tyrode's solution

(b) The peristaltic reflex in the rabbit's ileum

The reflex was initiated by raising the pressure in the lumen of the intestine by 2.5 to 4 centimetres In some preparations, in which the of saline increased pressure in the lumen was maintained, peristaltic waves continued for hours at regular intervals of 15 to 25 sec, interrupted from time to time by short periods of inactivity of the circular In other preparations cessation of the reflex took place within a few minutes after the pressure in the lumen had been raised. The procedure usually adopted was to raise the pressure at every 4th min for 1 min, and to keep it at zero or even below zero during the rest of the The reflex could then usually be initiated for hours without signs of fatigue, but in a few experiments the peristaltic reflex was not maintained even for the 1-min period and in others fatigue occurred in the course of a prolonged experiment. Some improvement was obtained by adding 1 in 100,000,000 to 200,000,000 eserine to the Tyrode's solution and by keeping the pressure in the lumen of the intestine at zero for 60 to 90 min before starting or continuing the experi-This procedure was adopted as a routine in all experiments in which it was not possible to obtain the peristaltic reflex regularly this procedure proved ineffective in a few preparations, which therefore had to be discarded

As stated by Trendelenburg (1917) the onset of the contraction of the circular muscle layer, which starts at the stomach and spreads to the caecalend, is preceded by shortening of the preparation owing to contraction of the longitudinal muscle fibres The degree of this shortening is dependent on the tone of the longitudinal muscle and varies greatly in different preparations. In addition we found that slight variations in the initial pressure in the lumen of the intestine greatly influenced the result With a negative pressure in the lumen the longitudinal muscle is well relaxed and, when the pressure is raised, it contracts strongly before the first wave of contraction of the circular muscle spreads over the preparation With an initial slight positive pressure in the lumen, the longitudinal muscle is already partly contracted and the additional rise in pressure leads to a relatively small further shortening of the preparation before it is stopped by the onset of the contraction of the circular muscle layer In Fig 4a and c the differ-

ences in the degrees of shortening in two preparations are illustrated, the initial pressure in the lumen at a was about zero, at c-1 cm saline

The fact that it is really the activity of the circular muscle which interupts the contraction of the longitudinal one can be seen from the results obtained when the pressure in the lumen was lowered at different phases of the peristaltic reflex. When this occurred during the end of a wave of contraction of the circular muscle there was no further shortening, but when it occurred in the interval between two peristaltic waves, when the longitudinal muscle had begun to contract, the shortening continued for some time after the pressure had been lowered. These differences are illustrated in the two controls shown in Fig. 7, before the administration of nupercaine.

With a negative pressure of 2 or 3 cm saline in the lumen of the intestine its walls are in apposition, the longitudinal muscle is well relaxed and exhibits no or only small rhythmic contractions

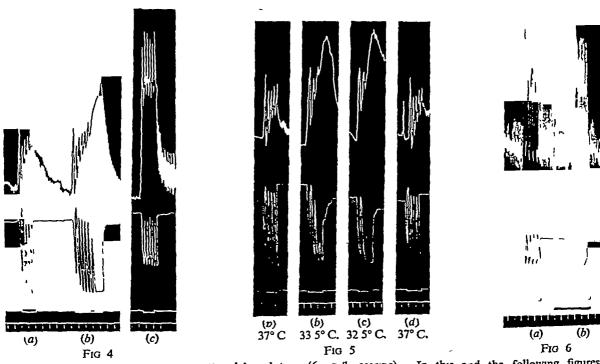


Fig 4—Rabbit's ileum in 35 cc Tyrode's solution (6 µg /1 eserine) In this and the following figures the upper tracing records contractions of the longitudinal muscle, the lower tracing the intestinal volume At (a), (b), and (c) pressure raised in intestinal lumen to about 3 cm saline during the periods indicated Time in 30 sec Details in text

Fig 5—Rabbit's ileum in 35 c c Tyrode's solution (6 µg /l eserine) Effect of temperature on peristaltic reflex initiated by raising pressure in intestinal lumen to about 3 cm saline for 1 min Time in 30 sec

Fig 6—Rabbit's ileum in 35 c c Tyrode's solution (6 µg/l eserine) Effect of cocaine on penstaltic reflex. Pressure raised in intestinal lumen to about 3 cm saline for 1 min at (a) and (b) Broad white line indicates presence of 100 µg cocaine in the bath Time in 30 sec Details in text

In this condition the preparation is particularly suitable for testing quantitatively the effects of drugs like acetylcholine on the longitudinal muscle. The first effect of a rise of pressure in the lumen is the reappearance, or an increase in the amplitude, of the spontaneous rhythmic contractions, with a further rise in pressure the tone of the longitudinal muscle increases

When fatigue of the peristaltic reflex takes place the response of the longitudinal muscle is not affected. The muscle either remains shortened or continues to do so after the contractions of the circular muscle have ceased (see Fig. 4b), and if the preparation had shown strong rhythmic contractions before the initiation of the reflex they reappear. The tracings then obtained resemble that illustrated for cocaine in Fig. 6

The effect of lowering the bath temperature is seen in the experiment of Fig 5. At 37° C a 1-min period of increased pressure in the intestinal lumen caused four peristaltic waves to spread over the whole length of the preparation (a and d), at 335° C there were two such waves and a third incomplete one (b), and at 32.5° C there occurred one full and one incomplete wave only (c). The contraction of the longitudinal muscle, however, was not inhibited when the bath temperature was lowered and the activity of the circular muscle had come to an end

Local anaesthetics - Von Anrep in 1880 and Bayliss and Starling in 1889 described inhibition

of the peristaltic reflex in the dog when cocaine was applied in strong concentrations to the serosa of the small intestine in situ According to Bayliss and Starling the spontaneous movements increased at the same time. On the isolated small intestine preparations of the rabbit and dog Trendelenburg (1917) obtained increased peristalsis with weak, but inhibition with strong, concentrations

In our experiments the main effect of cocaine was inhibition of the peristaltic reflex, although there was some indication of a stimulating action. The inhibition by

cocaine resembled the effect of fatigue or of lowering the bath temperature—ie, the contractions of the circular but not of the longitudinal muscle were affected (see Figs 6, 7, and 8) The concentrations of local anaesthetics necessary to inhibit the peristaltic reflex were of the same order as those which inhibit the response to small doses of nicotine

In the experiment of Fig 6 raising the pressure in the lumen of the intestine to 3 cm saline for 1 min produced four waves of contraction of the circular muscle layer (at a) At b 2 min after the addition of 100 µg of cocaine to the 35 cc bath these contractions no longer occurred when the pressure was raised, but the longitudinal muscle shortened and, in addition, exhibited its strong rhythmic contractions The Figure illustrates well the difference in the mechanisms responsible for the contractions of the two muscle layers when the pressure in the lumen is raised. The waves of contraction of the circular muscle are inhibited because a local nerve reflex is involved in their initiation. the spontaneous rhythmic contractions, the socalled pendular movements, and the increase in tone of the longitudinal muscle persist because they are myogenic in origin. In the experiment of Fig 7 raising the pressure in the lumen after the addition of 100 μ g cocaine to the bath still produced an initial small contraction of the circular muscle which affected the upper end of the preparation only, the longitudinal muscle, on the other hand, continued to contract during the whole period of increased pressure. The quick reversi-

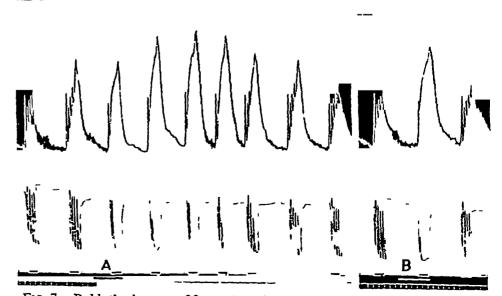


Fig 7—Rabbit's ileum in 35 c c Tyrode's solution (6 μg/l eserin) Comparison of 8 μg nupercaine (A) and 100 μg cocaine (B) on peristaltic reflex Pressure in intestinal lumen raised each time to about 3 cm saline for 1 min Broad white line indicates presence of local anaesthetics in bath Time in 30 sec

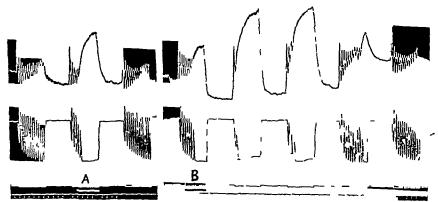


Fig. 8—Continuation of Fig. 6 Pressure in intestinal lumen raised for 3 min each time. Comparison of 100 µg cocaine (A) and 10 µg nupercaine (B) given 30 sec. after initiation of peristaltic reflex. Broad white lines indicate presence of local anaesthetics in bath. Time in 30 sec.

bility of the action of cocaine is seen from the fact that 3 min after the cocaine had been washed out the peristaltic reflex was restored, indeed, it often became more regular and any signs of fatigue originally present disappeared. For instance, in the experiment of Fig. 8A the last two waves of contraction of the circular muscle occurring during the first 3-min period of increased pressure in the lumen of the intestine were incomplete. After the cocaine had been washed out this "fatigue" was no longer seen. We do not know if this effect of cocaine is the result of increased excitability of the muscle fibres, or of the nervous elements, or of both

The inhibitory effect of cocaine develops quickly In the experiment of Fig 8A the addition of cocaine to the bath 30 sec after the peristaltic reflex had been initiated caused cessation of the peristaltic waves within 40 sec

Partial inhibition of the peristaltic reflex which occurs with small doses of cocaine shows the following characteristics (1) the contractions of the circular muscle layer come to an end after a short time, instead of the four or five waves usually passing over the preparation during a 1-min period of increased pressure there remain the initial two or three waves or even a single contraction only, (11) the waves of contraction become incomplete and confined to the upper end, or (111) they become irregular and start at the middle or lower end or at different points simultaneously, dilated and constricted segments then alternate with each other Such disorderly contractions are also frequently seen during the recovery period after a dose of cocaine greater than that necessary to produce complete inhibition of the peristaltic waves

When cocaine was used in concentrations so strong as to depress the excitability of the muscle fibres the response of the longitudinal muscle to the stimulus of increased pressure in the lumen was also abolished

The fact that the inhibiting action of cocaine on the peristaltic reflex is easily reversible, so that it is possible to obtain comparable effects on repeated administration of the same dose and graded responses with different

doses, makes it possible to employ this reaction for the biological assay of cocaine and to compare its action with that of other local anaesthetics When this was done it was found that procaine was slightly less active than cocaine in inhibiting the peristaltic reflex, whereas nupercaine was 12 to 124 times as active as cocaine and, in addition, the effect of nupercaine was more prolonged and developed more gradually In the experiment of Fig 7 the nupercaine was added to the bath 2 min before the pressure in the lumen was raised for 1 min and washed out after 3 min. This was the procedure usually adopted when assaying the potency of local anaesthetics against cocaine The greatest inhibition of the reflex did not occur during the presence of nupercaine in the bath but 3 min after it had been washed out, and about 20 min elapsed before the reflex was restored In the experiment of Fig 8, 8 µg nupercaine and 100 µg cocaine were added to the bath after the reflex had begun With cocaine it took about 40, with nupercaine about 70, sec before the reflex was inhibited, and again the prolonged effect of nupercaine was evident. This delay in the action of nupercaine was taken into account when assaying its potency against cocaine. The maximal inhibition observed after the nupercaine had been washed out was used for comparison

d-Tubocurarine chloride—This drug has only an inhibitory and no stimulating action on the peristaltic reflex. As with the inhibitory effect of cocaine only the waves of contractions of the circular muscle layer are abolished, whereas the rhythmic contractions of the longitudinal muscle, if present, and its tonic contraction in response to the increase in pressure in the lumen, persist this is illustrated in Fig. 9. In sensitive preparations

some inhibition of the reflex is seen with concentrations less than 1 in 10,000,000 (Fig 10), but for complete inhibition higher concentrations are necessary. The effect is easily reversible, recovery is delayed for more than a few minutes only when concentrations are used which are stronger than those necessary for complete inhibition of the reflex. Graded responses are obtained with different doses and comparable results with the same dose given repeatedly

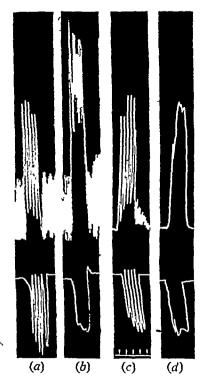


FIG 9—Rabbit's ileum in 35 c c Tyrode's solution. (6 μg/l eserine) The tracings (a) and (b) are from one, the tracings (c) and (d) from another, intestine Inhibition of peristaltic reflex by 100 μg d-tubocurarine chloride at (b) and (d), given 2 min before the initiation of the reflex Pressure in intestinal lumen raised each time for 1 min to about 3 cm saline. Time in 30 sec

The inhibition of the peristaltic reflex can be used as a reaction for assaying unknown solutions of tubocurarine and other substances with curarelike action. Fig. 10 illustrates the inhibition produced by different doses of d-tubocurarine chloride. With 100 and 50 μ g inhibition was complete, with 25 μ g one wave, with 10 μ g one and a half, with 5 μ g practically two, and with 2 μ g two and a half waves remained. Each dose of tubocurarine was added to the bath 2 min before the pressure was raised and washed out when the pressure was lowered again. The reflex was tested every 4th min. After the larger doses

of tubocurarine recovery was delayed, the reflex had not fully returned when it was tested 3 and 7 min after the tubocurarine had been washed out, it was retested only after full recovery had taken place, but with the exception of four controls, marked 0, all intermediate tests have been omitted in the Figure.

In the experiment of Fig 10 slight quick volume changes of the intestine occurred during the tubocurarine paralysis, their frequency was the same as that of the rhythmic contractions of the longitudinal muscle and they might in part have been accounted for in this way Partly, however, they resulted from the fact that the circular muscle layer itself exhibited rhythmic contractions at the lower end of the preparation, they were apparently myogenic in origin

(c) The peristaltic reflex of the guinea-pig's ileum

The reflex could be obtained regularly, repeatedly, and without the necessity of adding eserine to the bath fluid. A rise of pressure of 2 cm of saline in the lumen of the intestine was sufficient when the preparation was from a small, and of 2.5 cm when from a large, guinea-pig. During a 1-min period of increased pressure in the lumen, between 9 and 14 waves of contraction of the circular muscle layer passed over the preparation, and if the increased pressure was repeated every 4th min the preparation remained active for many hours, sometimes up to 10 hours

According to Trendelenburg cocaine had no stimulating effect on the peristaltic reflex of the guinea-pig's intestine when kept in the bath for 1 min cocaine had no effect at all, when kept for 5 min the reflex was abolished Our results were slightly different

Usually no definite inhibitory effect on the peristaltic reflex could be obtained with cocaine, nupercaine, or tubocurarine during the first hour or so of an experiment, even if the substances were tested in relatively high concentrations and kept in the bath for several minutes. But nearly all preparations gradually became more sensitive to the inhibiting action of these substances, and then inhibition occurred whether the substances were kept in the bath for 5 min or for 1 min. only

During the first hour or so of an experiment the characteristic effect of cocaine, and to a lesser degree of nupercaine and tubocurarine, consisted in an increase in the frequency of the waves of contraction of the circular muscle layer. In some preparations in which 1 in 40,000 cocaine had this effect at the beginning of the experiment a 10 times weaker concentration caused complete inhibition

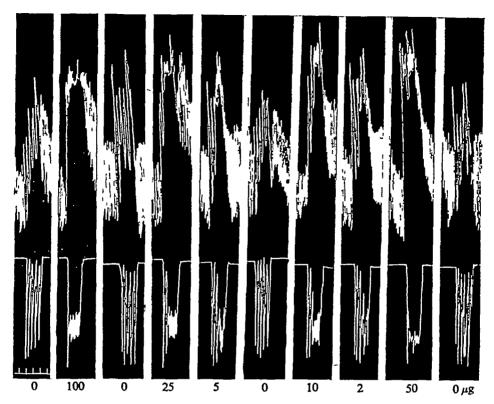


Fig 10—Rabbit's ileum in 35 cc Tyrode's solution (6 μ g/l eserine) Graded inhibition of peristaltic reflex by varying amounts of d-tubocurarine chloride Amounts indicated in μ g at bottom of figure Time in 30 sec Details in text

after the intestine had been in the bath for several hours. Although Trendelenburg does not mention this initial action of cocaine on the frequency of the peristaltic waves it is evident in an experiment which he illustrates in his paper.

In the experiments of Fig 11 the different actions of cocaine on the peristaltic reflex are Instead of the 12 peristaltic waves elicited by a 1-min period of increased pressure in the lumen at a, there were 20 waves when the procedure was repeated at b 2 min after the addition of 100 µg of cocaine to the 35 c c bath Sometimes up to 30 waves occurred. Each of these waves started at the upper end and extended over the whole preparation This was not always so, often they were incomplete, irregular, and started at different parts of the preparation, at the same time the longitudinal muscle contracted Such a result can be seen in the experiment of Fig 11 c to f, in which another intestine was used Instead of the 11 complete waves at c there were 18 incomplete waves along with shortening of the preparation at d At a later stage of the experiment

cocaine started to exert its inhibiting action and results like that seen at e were obtained an initial period of incomplete waves at a high frequency was followed by cessation of the reflex. At an even later stage of the experiment there was complete inhibition of the peristaltic waves after the addition of the same dose of cocaine. When it was washed out and the peristaltic reflex retested at 4-min intervals restoration of the reflex passed through a stage in which the frequency of the peristaltic waves was increased.

It was found difficult and sometimes impossible to obtain graded responses with increasing doses of cocaine and equal responses with the same dose, even at a later stage of an experiment, when cocaine exerted its strong inhibiting action on the peristaltic reflex. The isolated intestine of the guinea-pig, unlike that of the rabbit, therefore, is unsuitable for assaying the potency of an unknown solution of cocaine.

With nupercaine there were changes in the sensitivity of the preparation similar to those with cocaine, but it was usually possible to obtain some

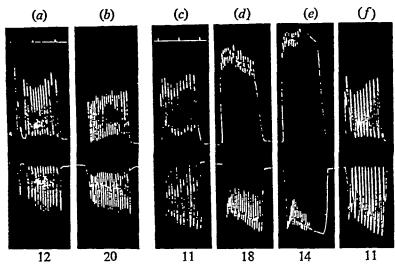


Fig 11 —Guinea-pig's ileum in 35 c c Tyrode's solution Effects of 100 μ g (at b) and 300 μ g (at d and e) of cocaine on the peristaltic reflex The number of peristaltic waves during each 1-min. period is given at the bottom Time in 30 sec Details in text

slight inhibition of the reflex even at the beginning of an experiment, therefore, when the potency of nupercaine was compared with that of cocaine no constant results could be obtained. In one preparation, for instance, $20~\mu g$ nupercaine

produced slight inhibition of the reflex at the beginning of the experiment whilst $800~\mu g$ cocaine only increased the frequency of the peristaltic waves, 4 hours later $100~\mu g$ cocaine as well as $20~\mu g$ nupercaine abolished the reflex In this experiment, therefore, nupercaine had an inhibiting action on the peristaltic reflex 40 times stronger than cocaine at the beginning of the experiment, but later on it was only five times as active

The increase in the frequency of the peristaltic waves produced by nupercaine was not so pronounced as with cocaine. When nupercaine had inhibited the reflex, recovery passed through a stage in which the frequency of the peristaltic waves was increased. As in the experiments on the rabbit's intestine the action of nupercaine was delayed and prolonged when it was kept in the bath for 3 min, the full effect only occurred a few minutes after it had been washed out.

At the beginning of an experiment tubocurarine, even in very strong concentrations, caused only slight acceleration of the frequency of the peristaltic waves, after several hours twentyfold weaker concentrations might abolish the reflex. The effects like those of cocaine were quickly reversible after washing out the drug. The difficulty of obtaining comparable responses on repeated administration of the same amount of tubocurarine, or graded responses on administration of varying amounts, made the guinea-pig's intestine preparation unsuitable for the quantitative assay of curare-like substances

In Fig 12 the changes in sensitivity to tubocurarine chloride are illustrated. The drug was added each time 2 min before the pressure in the lumen was raised. At the beginning of the experiment 2,000 μ g increased the frequency of the peristaltic waves from 10 to 13 per min (at b), $2\frac{1}{2}$ hours later 600 μ g greatly inhibited the reflex (at c), and

after a further $3\frac{1}{2}$ hours 100 μ g practically abolished it (at d)

The paralysing action of local anaesthetics and of tubocurarine on the peristaltic reflex is much weaker than that of nicotine in paralysing doses,

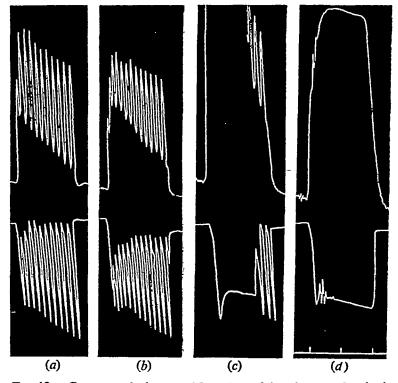


FIG 12 —Guinea-pig's ileum in 35 c c Tyrode's solution Gradual increase in the inhibition of the peristaltic reflex by d-tubo-curarine chloride At (b) 2,000 μ g., at (c) 600 μ g two and a half hours later, at (d) 100 μ g six hours later Time in 30 sec Details in text

particularly when the comparison is made on a fresh preparation. An analogous result has been obtained and referred to previously for the inhibition of the stimulating action of nicotine.

In the experiment of Fig. 13, 2,000 ug of dtubocurarine chloride had no inhibiting action. 600 µg of nicotine, however, abolished the reflex The nicotine was added to the bath between a and b whilst the pressure in the intestinal lumen was low, an immediate strong contraction of the longitudinal muscle occurred, but within 1 min the muscle relaxed again although the nicotine was kept in the bath At this stage raising the pressure in the lumen no longer initiated the peristaltic reflex (at b) In fact, both the longitudinal and the circular muscle layer were in an atonic condi-Instead of the usual strong contraction of the longitudinal muscle in response to the increased pressure in the lumen there was only a slight delayed shortening of the preparation, and the lack of tone of the circular muscle led to the great filling of the intestine with saline solution seen on the volume record by the profound fall which is followed by a horizontal line because the maximal possible reduction in volume of the Brodie recorder has been reached Between b and c the nicotine was washed out, before the reflex returned, the muscle tone was restored in both layers With the return of the reflex the peristaltic _ contractions of the circular muscle were at first not

Fig. 13—Guinea-pig s ileum in 35 c c Tyrode's solution Nicotine paralysis of the peristaltic reflex Between (a) and (b) 600 µg nicotine tartrate were given and washed out at the end of (b) Time in 30 sec. Details in text

sufficiently strong to overcome the contraction of the longitudinal muscle as will be seen from a comparison of e with a and f

If the nicotine had remained in the bath the return of muscle tone seen at c and d in Fig 13 would nevertheless have occurred but the abolition of the peristaltic waves would have remained. In the early stages of nicotine paralysis, but not later, the muscle layers are thus unable to develop tone in response to increased pressure in the lumen and in this condition they have also been found to be less excitable to drugs like acetylcholine or histamine (Cantoni and Eastman, 1946, Emmelin and Feldberg, 1947)

DISCUSSION

On the isolated intestinal preparation it can easily be shown that the response of the intestinal wall to increased pressure in the lumen consists of two phases a contraction of the longitudinal muscle and a wave of contraction of the circular muscle spreading aborally over the whole preparation. Trendelenburg has aptly termed the two responses the preparatory and the emptying phase of the peristaltic reflex. Only the peristaltic wave of contraction of the circular muscle is nervous in origin and therefore very susceptible to lowering of the bath temperature, to fatigue, and to drugs which affect nerve fibres or nerve cells in the intestinal wall. The contraction of the longitudinal muscle, on the other hand, is a response of the

muscle fibres themselves to the stimulus of stretching and is therefore not abolished when the function of the nervous structures in the intestinal wall is interfered with by anaesthetics or by tubolocal In the rabbit's intestine curarine rhythmic with pronounced ıts contractions of the longitudinal muscle there occurs in addition, even before the pressure in the lumen is sufficiently raised to initiate the shortening of the preparation, an augmentation of the amplitude of the rhythmic contractions response also is myogenic in origin We do not know whether similar myogenic responses to stretching occur in the circular muscle layer This muscle certainly offers some resistance to the stimulus of filling the gut, and this resistance is not abolished when the nervous structures in the intestinal-wall are inactivated by cocaine or tubocurarine, but it is abolished when the muscle fibres are in an atonic condition as, for instance, in the early stages of nicotine paralysis. In addition we have found* that the whole layer of the circular muscle may be thrown into powerful rhythmic contractions at the same frequency as that of the rhythmic contractions of the longitudinal muscle, when eserine in a concentration of about 1 in 300,000 is added to the bath during a period of increased pressure in the lumen and whilst the peristaltic waves of contraction are abolished by cocaine or tubocurarine

A study of the peristaltic reflex as well as of the simple contractions of the longitudinal muscle on the intestinal preparation reveals the fact that a careful analysis is always necessary before the effects of drugs can be attributed to a nervous or to a muscular site of action For instance, the shortening of the intestinal preparation when the ganglion cells are stimulated by nicotine does not differ much from the contraction produced by muscle-stimulating drugs, such as histamine or acetylcholine On the other hand, if the pressure exerted on the intestinal wall from the lumen is just insufficient to initiate the reflex, any drug which increases muscle tone may indirectly elicit the reflex It is therefore not surprising that drugs which stimulate smooth muscle are known to augment, whereas drugs which relax them are known to inhibit, peristalsis Straub and his co-workers applied Trendelenburg's technique to the intestine in situ and observed increased peristalsis with choline, acetylcholine, and physostigmine, and inhibition of the peristaltic reflex with adrenaline and ephedrine (Straub and Viaud, 1933, Straub and Leo, 1933, Leo, 1933, Straub and Stefánsson, 1937)

If drugs are known to have local anaesthetic or curare-like actions the abolition of the peristaltic reflex in the isolated rabbit's intestine provides a useful reaction for assaying their potency, provided that the concentrations used do not affect the muscle fibres. Nupercaine was found to be about 12 times as active as cocaine in abolishing the peristaltic reflex, this does not necessarily prove a similar relationship for the potency of the two drugs as local anaesthetics, since with different tests widely different results are obtained (see Goodman and Gilman, 1943)

When the peristaltic reflex is used as a reaction for assaying curare-like substances we compare their effectiveness in paralysing autonomic ganglia and not motor endplates. The two effects do not run parallel, as shown by Bovet, Depierre, and de

Lestrange (1947) and by Depierre (1947) In previous experiments of this kind the paralysing effects on sympathetic ganglia were compared with those on motor endplates. The ganglia involved in the peristaltic reflex, however, probably belong to the parasympathetic nervous system, and their sensitivity to a curare-like acting substance may be different from that of sympathetic ganglia. The problem appears to be even more complicated since motor endplates of muscles from different species do not show the same sensitivity to different curare-like substances (Wien, 1948)

The greater frequency of the peristaltic waves after cocaine in the freshly suspended guinea-pig's intestine is difficult to explain. When at the same time the waves become incomplete, so that they affect parts of the preparation only, they resemble rhythmic contractions of myogenic origin they are not accounted for in this way, for the following reasons (1) All grades of increased frequency may occur after cocaine The frequency of the contractions may increase slightly or the rate may double or increase even more If cocaine were to abolish the peristaltic waves and reveal myogenic contractions there would be only two frequencies—that of the peristaltic waves before, and that of the myogenic contractions after, cocaine (2) When the contractions do not become incomplete after cocaine, they, like typical peristaltic waves, start at the oral end and spread over the whole preparation

The frequency of the peristaltic contractions is to some extent dependent on the degree of pressure exerted in the intestinal lumen and increases with the pressure (Trendelenburg, 1917) might be thought to produce a similar effect in the guinea-pig's intestine simply by increasing musclè tone It is more likely, however, that cocaine, and to some extent other local anaesthetics, alter the excitability or the conductivity of either the muscle fibres or the nervous elements or of both structures in the intestinal wall of the guinea-pig in such a way as to speed up the rate of the rhythmic response which occurs when the pressure in the lumen is increased This action could be grouped then with the well-known central stimulating actions of cocaine. To assume a similar action for tubocurarine would be strange, but its effect in increasing the frequency of the peristaltic waves is only very weak and it may have to be explained in a different way

SUMMARY

1 Cocaine has stimulating and inhibiting effects on the excitability of the muscle fibres of the rabbit's and guinea-pig's intestine d-Tubocurarine has but slight effects of this kind on these preparations

- 2 It is possible to inactivate the nervous structures in the intestinal wall with cocaine or with d-tubocurarine. In concentrations which do not yet depress the excitability of the muscle fibres these substances inhibit the responses to small doses of nicotine as well as the peristaltic reflex
- 3 The peristaltic reflex initiated by raising the pressure in the lumen of the isolated intestine (Trendelenburg's method) consists of two phases (1) a contraction of the longitudinal muscle which is a response of the muscle fibres themselves to the stimulus of stretching, and (ii) waves of contractions of the circular muscle layer spreading aborally over the whole preparation and being nervous in origin Only the latter are abolished when the nervous structures in the intestinal wall are paralysed by local anaesthetics or by tubocurarine In the rabbit's intestine the familiar strong rhythmic contractions of the longitudinal muscle (pendular movements) also remain unaffected under these conditions
- 4 In the guinea-pig's intestine local anaesthetics and d-tubocurarine exert their inhibiting effect on the peristaltic reflex only several hours after the preparation has been suspended in the bath. In the freshly suspended preparation the effect of these drugs is to increase the number of peristaltic waves per minute. This effect is strong with cocaine but weak with tubocurarine

5 Inhibition of the peristaltic reflex in the isolated rabbit's intestine provides a useful reaction for assaying quantitatively the potency of local anaesthetics or curare-like substances, because the inhibition is easily reversible, and comparable effects are obtained on repeated administration of the same dose of these substances, and graded responses with different doses. By this method nupercaine was found to be about 12½ times as active as cocaine.

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THE EFFECT OF N-METHYLATION ON CERTAIN IMINAZOLINES

BY

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In a previous paper (Gowdey, 1948) it has been shown that methylation of a nitrogen atom in the iminazoline ring of 2-benzyliminazoline (BI) produces important changes in its pharmacological activity. Thus the N-methyl derivative, instead of causing a fall of blood pressure through peripheral vasodilatation, caused a rise of blood pressure, but had no direct effect on vessels, and the pressor effect was shown to be due to a nicotine-like liberation of adrenaline from the adrenals. On the other

TABLE I

CH₂—CN—CH₂

N—CH₂

H

2-benzyliminazoline (BI)

N-methyl-2-(1'-naphthylmethyl)ımınazolıne (MeNI)

$$\begin{array}{c|c} & & \\ & &$$

N-methyl-2-(N-benzylanılınomethyl)ımınazolıne (MeBAI)

N-methyl-2-(p-methoxybenzyl)iminazoline (MeMeOBI)

hand, N-methylbenzyliminazoline had the same qualitative action as BI itself on cardiac tissue, the intestine, skeletal muscle, and the superior cervical ganglion

Because of the sudden appearance of a nicotine-like vascular activity of the BI molecule when it contained an N-methyl group, it was decided to compare several more 2-substituted iminazolines with their N-methyl derivatives. The structural formulae of the compounds investigated, and the abbreviations which will be used in referring to them, are given in Table I

The vascular activity of 2-(1'-naphthylmethyl)-iminazoline (NI) has been studied by several workers (Hartmann and Isler, 1939, Meier and Muller, 1941, Emerson, 1944, Craver et al, 1944, Yonkman et al, 1945, Meier and Bucher, 1946a) 2-(N-Benzylanilinomethyl)iminazoline (BAI), although studied mainly as an antihistamine compound, was tested for other activities by Meier and Bucher (1946b) and by Craver et al (1948) p-Methoxybenzyliminazoline (MeOBI) was investigated by Hartmann and Isler in their early paper on the iminazoline series (1939) None of the N-methyl derivatives except MeBI has been mentioned in the literature

EXPERIMENTAL RESULTS

A Action on the cardiovascular system

(1) Naphthylmethylminazoline (NI) and MeNI—The intravenous injection of 25-100 µg NI into a spinal cat causes a large rise of blood pressure, but no change in heart rate. The same dose repeated several times causes less rise of blood pressure than the first dose. The pressor effect of NI is not abolished but it may be reduced by large doses of nicotine or tetraethylammonium iodide or by adrenalectomy. Fig. 1 shows the large rise of blood pressure of the spinal cat produced by the injection of 1 mg. NI at C compared to the rise produced by 20 µg adrenaline at A

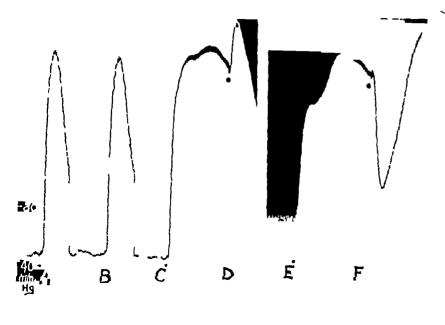


Fig 1 —Spinal cat Injections A, B, 20 μg. adrenaline, C, 1 mg NI, D, 20 μg adrenaline, E, 20 mg NI, F, 20 μg adrenaline

and B It also shows that a large dose (20 mg) of NI (injected at E) reversed the pressor action of 20 μ g adrenaline (injected at F). Thus NI, although a pressor substance, reverses the action of adrenaline in large doses. Both ergotoxine and BI abolished the rise of blood pressure caused by NI, but neither reversed it

Doses of 25-100 µg MeNI have no appreciable effect on the blood pressure, but large doses (1-2 mg) cause a rapid rise accompanied by an increased heart rate. That this pressor action of MeNI was due to a nicotine-like liberation of adrenaline was shown both in the spinal cat and in the cat under chloralose anaesthesia. Large doses of nicotine abolished this pressor response It was also found that large doses of MeNI (5-10 mg) injected during the "nicotine paralysis" caused a fall of blood pressure which was later shown to be due to cardiac depression

Unlike BI and MeBI which stimulated the isolated perfused cat's heart, NI and MeNI depressed the heart Fig 2 (top) shows the effect of injecting 0.25 mg NI at the 2nd arrow, the heart rate decreased by eight beats/min, the coronary flow from 5.2 to 4.2 ml/min, and the force of contraction by 40 per cent. At the 1st arrow 0.25 mg MeNI caused a similar decrease in heart rate and coronary flow, but less reduction in

amplitude In the isolated perfused rabbit's heart NI caused great depression, but in the dog heart-lung preparation injections up to 10 mg had no appreciable effect on cardiac rate, output, or volume

NI caused vasoconstriction in the rabbit ear perfused by Locke's solution. and in the dog's hindleg perfused with blood containing heparin by means of a Dale-Schuster pump The powerful vasocontrictor action of NI is absent in its N-methyl dérivative Thus in the perfused rabbit's ear 2 mg MeNI had no effect, whereas 2 μg NI caused a long-lasting vasoconstriction Thus the unsubstituted compound is

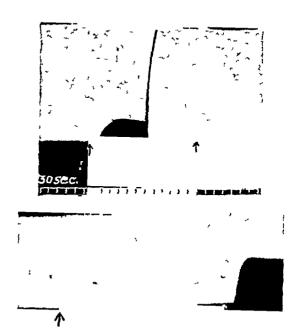


Fig 2—Cat heart, Langendorff preparation Upper record, 1st arrow, 0.25 mg MeNI, 2nd arrow, 0.25 mg NI Lower record, 1st arrow, 0.25 mg MeBAI, 2nd arrow, 0.25 mg BAI

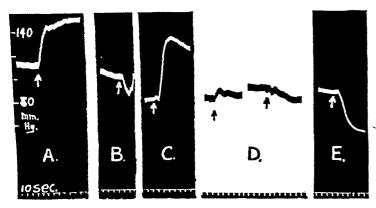


Fig 3—Cat, chloralose A, 5 mg MeMeOBI, B, 10 mg MeOBI, C, 2 mg MeBAI, between C and D, 19 mg nicotine acid tartrate injected, in D, first 5 mg MeMeOBI, second 2 mg MeBAI E, 2 mg BAI

over 1,000 times as active a vasoconstrictor as its N-methyl derivative in this preparation

(2) Benzylanilmomethyliminazoline (BAI) and MeBAI—Fig 3C shows the abrupt rise of the cat's blood pressure produced by injecting 2 mg MeBAI, the same dose of the unsubstituted compound injected at E caused a long-lasting depressor response After large doses of nicotine the pressor response to 2 mg MeBAI (injected at 2nd arrow in D) was abolished. Thus it was concluded that the rise of blood pressure caused by MeBAI was also a nicotine-like effect.

Both BAI and MeBAI depressed the isolated perfused cat's heart. The effect of injecting 0.25 mg of those compounds is shown in Fig. 2 (bottom). The reduction in heart rate and force of contraction was similar to that caused by the naphthyl compounds, but the coronary flow was first increased by the benzylanilino compounds before the longer-lasting reduction occurred.

In the isolated perfused rabbit's ear it was found that the injection of 1 mg BAI or MeBAI produced definite vasodilatation. The N-methyl compound seemed somewhat weaker in this respect than the parent substance

(3) p-Methoxybenzyliminazoline (MeOBI) and MeMeOBI—These compounds will be seen (Fig 3) to have the same general action on the cat's blood pressure as BAI and MeBAI. Thus 5 mg MeMeOBI injected at A caused a rise of blood pressure and increased the heart rate, this pressor response was no longer significant when the same dose was injected, at the first arrow in D, after nicotine, 10 mg MeOBI caused a fall of blood pressure when injected at B. The depressor action of MeOBI is not the same as that of BAI, however, because it was found that 10–20 mg MeOBI injected into a spinal cat reversed the pressor effect

of small doses of adrenaline in the same way as BI had done Further experiments showed that very large doses of adrenaline caused a pressor effect after MeOBI as they had been shown to do after BI

The isolated perfused cat heart was depressed by both MeOBI and its N-methyl derivative. In this respect the methoxybenzyl compounds were unlike BI and MeBI

B Action on the intestine—The normal tone of the isolated guinea-pig ileum was reduced by NI, BAI, and MeBAI, by MeOBI, and MeMeOBI These compounds also antagonized the contractions induced by acetylcholine NI in a concentration

of 4×10^6 was shown to reduce greatly the histamine-induced iteal contraction (see Fig 4A) On the other hand, MeNI (5×10^{-7}) produced a slowly developing contraction of the iteum which was slightly less than that caused by acetylcholine ($2 \times 10^{\circ}$) If an insufficient interval was left between doses of MeNI, the stimulating

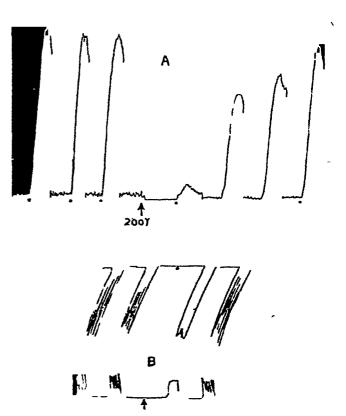


Fig 4 —Guinea-pig ileum, 50 ml bath Locke's solution A 0.2 μ g histamine at each dot, 0.2 mg NI at the arrow B Trendelenburg preparation, upper record showing peristalsis, lower record longitudinal contractions. At arrow MeMeOBI was added to bath so that the concentration was 4 \times 10 5 , addition being made 2 min before the next stimulus

effect was reduced or lost completely, presumably because nicotine-like paralysis occurred

The pendulum movements of the isolated rabbit's duodenum were inhibited by NI in a concentration of 10^{-7} , about ten times the dose of adrenaline required to produce the same effect MeNI in a concentration of 2×10^{-6} increased the tone and rhythm of the intestine, and prevented the inhibitions regularly caused by 4×10^{-6} NI

In the Trendelenburg peristalsis preparation as modified by Feldberg and Lin (1948) NI was found to have no effect on the peristalsis, but it inhibited the longitudinal contractions of the ileum BAI, MeOBI, and its N-methyl derivative inhibited both sets of muscle fibres. The inhibitory effect of 4×10^{-5} MeMeOBI on the circular and longitudinal muscles of the ileum is shown in Fig. 4B

C Action on skeletal muscle—All these immazolines, substituted or not, caused a curariform block in the transmission of the nerve impulse in the phrenic nerve-diaphragm preparation of the rat

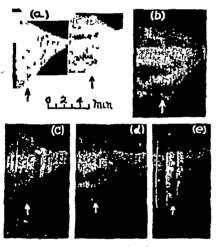


FIG 5—Rat phrenic nerve-diaphragm preparation Stimulation with maximal shocks 6-12/min Bath 50 ml T = 36° C (a) 1st arrow, 10 mg NI, 2nd arrow, 10 mg MeNI (b) 10 mg BAI (c) 10 mg MeMeOBI (d) 10 mg MeOBI (e) MeBAI All substances allowed to act for 5 min

Fig 5 illustrates this action, all the compounds were given in a final concentration of 2×10^4 Although slight differences in potency were observed, the curarizing effects of these compounds were all of the same order of magnitude

Curariform activity was also observed in the sciatic-gastrocnemius preparation of the cat Fig 6 shows the decreased muscle tension produced by the intra-arterial injection of the various N-methyl

derivatives The parent compounds have the same curarizing action and with doses of the same order of magnitude. That this was a true curariform action and not a direct effect on the nerve or on the muscle was shown by the following experiments. The intra-arterial injection of a large dose

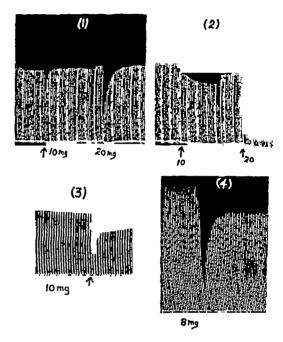


FIG 6—Cat, chloralose, left sciatic-gastrocnemius preparation, record of gastrocnemius contraction with tension lever Injections made into aorta at the bifurcation through the stump of the right external iliac artery (1) 10 mg and 20 mg MeMeOBI, (2) 10 mg and 20 mg MeBAI, (3) 10 mg MeNI, (4) 8 mg MeBI

(20 mg) of MeNI into the fully curarized, directly stimulated gastrocnemius of the cat did not alter the tension developed in the muscle. Nor did MeNI in a concentration of 10 mg/ml applied directly to the nerve of the isolated frog's nervemuscle preparation cause any change in the twitch tension evoked by stimulation through the nerve

D Action on the perfused superior cervical ganglion—Since the N-methyl compounds of this series shared the nicotine-like pressor action of MeBI, it was expected that they might, like MeBI and nicotine, stimulate the ganglion before depressing its response to preganglionic stimulation. But although depression or abolition of ganglionic transmission occurred with all the compounds, N-methyl-substituted or not, none of them caused a stimulation of the ganglion cells on injection as MeBI had done. A typical example of the curari-

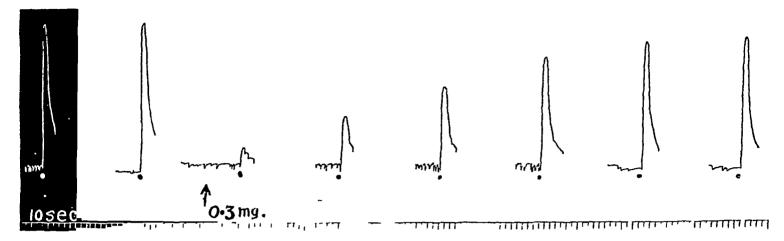


Fig 7—Cat, chloralose Right superior cervical ganglion perfused. Contractions of nictitating membrane in response to supra-maximal stimulation of preganglionic fibres. At arrow 0.3 mg. MeMeOBI injected into the fluid perfusing the ganglion. Note depression of effect of subsequent stimulation.

form block in transmission is shown in Fig 7, 03 mg MeMeOBI had no effect by itself, but decreased the contraction of the nictitating membrane caused by maximal preganglionic stimulation

DISCUSSION

The striking thing about this series of iminazolines is that, regardless of the actions of the unsubstituted compounds, their N-methyl derivatives without exception cause a rapid rise of blood pressure and an increased heart rate The pressor effects have been observed in spinal cats and in pithed cats, therefore the action is not due to stimulation of centres in the cord or brain this and the fact that large doses of nicotine abolish the blood-pressure rise, it is concluded that the N-methyliminazolines liberate adrenaline from the The pressor action does not appear to depend on a simultaneous stimulation of sympathetic ganglia, for when these substances are injected into the perfused superior cervical ganglion they do not exert a stimulant effect like nicotine (Feldberg and Vartiainen, 1934), BI, and MeBI (Gowdey, 1948) Other substances such as pilocarpine and histamine are already known to liberate adrenaline from the suprarenal glands without stimulating the sympathetic ganglia at the same time The action of the iminazolines on the ganglion therefore resembles that of tubocurarine rather than that of nicotine, and it is interesting that the curariform activity of the N-methyl compounds is shared by the unsubstituted compounds, as that of nicotine is shared by lobeline, cytisine, etc Ganglionic transmission is also blocked by the tetraethylammonium ion as well as by tubocurarine and the iminazolines, but the nerve-muscle experiments again suggest that the iminazolines are acting more like tubocurarine than like tetraethylammonium, since the latter augments the contractions of the rat diaphragm (Barlow and Ing, 1948) whereas tubocurarine and the iminazolines alike reduce them

Of this series only BI and MeBI stimulate the isolated cat heart, the other compounds have a depressant action in the same dose range. In the isolated vessels of the rabbit's ear BI produces vasodilatation, NI produces vasoconstriction, yet their N-methyl derivatives have no effect in a dose 1,000 times as great. Thus N-methylation of the 2-iminazolines causes a shift in their site of action, so that their effect on the blood pressure is now not a direct effect on the peripheral vascular system but is the resultant of the liberation of adrenaline and their direct action on the heart

Although MeNI stimulated the isolated intestine, the other compounds of this series, whether substituted or not, caused inhibition of the tone, and of the contractions induced by acetylcholine Experiments with the Trendelenburg preparation showed that the benzylanilinomethyl and pmethoxybenzyl compounds also prevented peristalsis Feldberg and Lin (1948) showed that tubocurarine abolishes peristalsis in this preparation, and since these compounds have a curariform action the inhibition they cause may be due to this property

SUMMARY

The actions of 2-(1'-naphthylmethyl)iminazoline 2-(N-benzylandinomethyl)iminazoline, and p-methoxybenzyliminazoline on the blood pressure.

on the isolated heart and perfused vessels, on the intestine, and on ganglionic and neuromuscular transmission have been compared with those of their N-methyl derivatives

N-methylation was found to cause a shift in the site of action so that the-effect of these compounds on the blood pressure was not due to a direct action on the peripheral vascular system but was the resultant of more central actions stimulation of sympathetic ganglia, liberation of adrenaline, and direct effect on the cardiac muscle

Nicotine-like activity on the isolated intestine was shown only by N-methyl-(naphthylmethyl)-iminazoline, the other compounds inhibited the tone, and antagonized the contractions evoked by acetylcholine (N-Benzylanilinomethyl)iminazoline, p-methoxybenzyliminazoline, and their N-methyl derivatives had a curariform effect on intestinal peristalsis

All the compounds of this series, whether N-methylated or not, showed curariform activity on the superior cervical ganglion and on neuro-muscular transmission

I am indebted to Dr W F Short and Dr P Oxley, of Boots Pure Drug Co, for the compounds used in this study, I also wish to thank Professor J H Burn and Dr G S Dawes for their guidance and encouragement

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THE TOXICITY OF VESICANTS AND SOME OTHER COMPOUNDS TO THE PYRUVATE OXIDASE SYSTEM* (BRAIN)

BY

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The pyruvate oxidase system in pigeon brain tissue is known to be delicate and sensitive to the tissue inhibitors fluoride, iodoacetate, and phloridzin (Peters and Sinclair, 1934, Peters, Rydin, and Thompson, 1935) During the war it was used as a test system in Chemical Defence Research during the course of a planned investigation, the origin and trend of which has been described elsewhere (Peters, 1947) The fundamental postulates were that vesication could be initiated by damage to an enzyme with the pyruvate oxidase system as a leading string, and that the attack was upon a sulphydryl grouping, these originated in the earlier finding (Peters, 1936) that dichlorodiethylsulphone (HO₂) (as well as arsenite) had a selective action on the lactate oxidase system in brain, poisoning it at the pyruvate stage There was a close analogy with the action of iodoacetate, also a vesicant † At the outbreak of war the researches described here developed simultaneously with the arsenical investigation (Peters, Stocken, and Thompson, 1945) by a team which included E Holiday A G Ogston, J St Philpot, and L A Stocken the objective was a better understanding of the vesicant action and the hope of more effective therapy

In examining the suitability of the pyruvate oxidase system in brain as a test enzyme its

behaviour to several substances allied to mustard gas (H) was carefully investigated, some comparisons were also made with other enzymes. It was found that there was some correspondence between the toxicity of many non-arsenical vesicants to the pyruvate oxidase system and their potency as vesicants, but that this relation failed for H, even so, the enzyme system was still sufficiently sensitive to the latter to be used as a test agent It is interesting to note in regard to our observations (Peters and Wakelin, 1946) made also at this time upon the -SH nature of some components of the pyruvate system that G Barron (1936), in the course of observations upon the inhibitors of pyruvate oxidase from gonococcus. noted its sensitivity to oxygen, also more recently Mann and Quastel (1946) and Dickens (1946) consider that it is the enzyme preferentially attacked in high pressure oxygen poisoning. When taken together this work and that upon the biochemical lesion in aneurin deficiency and in arsenical poisoning from this laboratory emphasize the pharmacological importance of this enzyme system

The present paper embodies the gist of 5 reports written early in the war (Peters and Wakelin, 1940, 1941) and is divided into sections upon the water soluble and water insoluble substances and observations upon other enzymes

I WATER SOLUBLE SUBSTANCES

Experimental methods

Brain brei and dispersion and pyruvate dehydrogenase— These were prepared as described previously (Kinnersley, O'Brien, and Peters, 1935, Banga, Ochoa, and Peters, 1939, Peters and Wakelin, 1946) Brei and

^{*}This paper was planned to be the initial publication of a series which includes two by the same authors elsewhere (Blochem J 1946 40 513 and 1947, 41 545) its publication has been delayed through unforeseen circumstances. Though some figures are included which are given in one of the other papers it has been thought desirable to publish the data upon which they are based, because they indicate the kind of accuracy obtainable in this type of pharmacological enzyme investigation.

[†] Berenblum Kendall, and Orr (1936) found that mustard gas was more toxic to the whole glycolytic process in tissue than to respiration

slice experiments were made with oxygen in the bottles, and dispersion experiments with air

Experiments with the pyruvate oxidase system in pigeon's brain tissue can be made with tissue prepared in three different ways, as slices, as brei (a mashed preparation), and as a dispersion, made by grinding the brain finely in a mortar under ice-cold conditions. The slice resembles the organized tissue best in this H M Carleton has shown that the cell outlines are still intact (unpublished results) In the brei the cell outlines have been mainly destroyed, though large numbers of nuclei are still intact though the brei approximates more to a preparation of enzymes, some phosphoric esters still fail to penetrate to the active centres present, for instance, vitamin B, will penetrate, whereas its pyrophosphoric ester (cocarboxylase) will not (Peters, 1937) dispersions we have an enzyme system sufficiently homogeneous to reduce permeability phenomena to a minimum, this has been produced, however, at the expense of stability and a dispersion is only stable for about 30 min at 38°, it can therefore be used only for rapidly acting substances It is not necessarily identical with the so-called "homogenates" (Potter and Elvehjem, 1936, Elliott, Scott, and Libet, 1942) As several of the poisons concerned react rather slowly, the most useful information was obtained with the more stable "brei"

Any uncertainties produced by the residual respiration could have been largely eliminated, if desired, by a preliminary washing of the brei with ice-cold Ringer-phosphate solution (Long and Peters, 1939), the delay which would have been caused by this refinement would have made little difference to the result and was not

thought worth while Most experiments were therefore made with unwashed brei

Respiration was studied in Barcroft Dixon or Warburg type respirometers in Ringer-phosphate pH 7 3, 3 0 ml per bottle \pm Na pyruvate (1 8 \times 10-2 ^{2}M), the poison was added last after division of the tissue (approx 100 mg pigeon brain "brei") Each figure quoted in Table I and elsewhere represents the average rate of respiration in μ l/g/hr moist tissue (water content approx 80 per cent) for the time interval given, excluding the initial period of 12 min required for equilibration Temp 38° Gas O₂ or air One pigeon brain (cere brum + optic lobes) can be distributed conveniently among 12 bottles In this series 8 bottles (4 duplicates) were devoted to the values for pyruvate alone and those of pyruvate + poison, and the remaining 4 were used to obtain residual values

A typical experiment is given (Exp 1939) in Table I, it confirms the previous finding (Peters, 1936) that HO₂ has a marked toxic action upon this system, and that there is no protection even with a relatively high concentration of vitamin B₁ (1 μ g would be a maximum dose for an avitaminous brain). It will be noticed that the effect of HO₂ appears slowly with these concentrations, even with 100 μ g/3 ml not until after 15 min have elapsed

The falling rate of respiration is due largely to the decrease in residual respiration much work (Peters, 1938) has shown that the extra rate of respiration over the period 30–120 min forms the most reliable estimate in experiments of this type. This period was therefore used to compare the inhibitory effects

= TABLE I = FFECT OF HO₂ (100 μ g = 675 μ M (10-6M) upon the oxygen uptake of pigeon brain brei in ringer-phosphate pH 7 3 substrate Na pyruvate (0 018M)

Exp conditions	O ₃ uptake in μl /g /hr during respiration periods (min)			Ayerage			
	0–15	15-30	30-60	60-90	90–120	0-30	30-120
(a) No addition (b) + 50 μg HO ₂ * (c) + 100 μg HO ₂ *	1550 1405 1280	1276 1090 1040	903 750 640	790 437 246	490 222 162		
(d) Na pyr (e) Na pyr + 50 \(mu_g\) HO ₂ (f) Na pyr + 100 \(mu_g\) HO ₂ (g) Same + 10 mg vit B ₁	3315 3285 3130 3185	3060 2875 2340 2465	2710 2080 1450 1623	2305 1325 764 917	1845 956 361 431		
DIFFERENCES FOR Pyr alone (d) – (a) Same + 50 μ g HO ₂ (e) – (b) Same + 100 μ g HO ₂ (f) – (c)	1765 1880 1850	1784 1785 1300	1807 1330 810	1515 888 518	1355 734 199	1780 1832 1675	1559 984 509

^{*} Single observations. Remainder are average duplicates HO₃ = Dichlorodiethylsulphone

50 μg HO₂ 100 μg HO₂

RESULTS

Table II gives the results obtained for a number of water soluble substances The 50 per cent inhibition values (ET50, expressed in μM) were estimated by inspection of curves drawn through the known points

In ordinary experiments of this type the standard deviation of a single observation is c 3 5 per cent, but in the present work substantial further errors undoubtedly arose from difficulties in getting satisfactory solution of some of the substances used, and from uneven actions of the poisons in the early stages before the brei was properly divided

TABLE II

SUMMARY OF AVERAGE PERCENTAGE INHIBITIONS IN OXYGEN UPTAKE OF PIGEON BRAIN BREI PRODUCED BY SEVERAL WATER SOLUBLE VESICANTS AND OTHER SUBSTANCES SUBSTRATE Na PYRUVATE (0 018M)

Respiration period 30-120 min No of experiments in brackets

Conc <i>M</i> × 10-4	% change	μM for 50% in-
0 437 0 875 1 75	-22 7 -48 3 -69 4	90
1 91 4 77 9 55	-35 9 -55 1 -63 7	380*
0 29 0 86 2 66	-3 2 -52 7 -92 5	75
1 9 5.72 15 2 57 2 95 2	+65 +7 +65 -4 -11	Nil at 572
3 3 4 07 8 15 16 3	+1 -25 2 -48 -61	900
2 16 4 32 5 4	-46 8 -69 5 -60 3	250
0 54 1 08 1 78	-51 8 -67 7 -85 7	50
55 and 43	Nil	Nil at
	0 437 0 875 1 75 1 91 4 77 9 55 0 29 0 86 2 66 1 9 5.72 15 2 57 2 95 2 3 3 4 07 8 15 16 3 2 16 4 32 5 4 0 54 1 08 1 78	M×10-4 change 0 437 -22 7 0 875 -48 3 1 75 -69 4 1 91 -35 9 4 77 -55 1 9 55 -63 7 0 29 -3 2 0 86 -52 7 2 66 -92 5 1 9 +6 5 57 2 +6 5 57 2 -4 95 2 -11 3 3 +1 4 07 -25 2 8 15 -48 16 3 -61 2 16 -46 8 4 32 -69 5 5 4 -60 3 0 54 -51 8 1 08 -67 7 1 78 -85 7 55 and Nil 43 Nil

^{*}The methyl bis(2-chloroethyl)amine was found by Peters, Thompson and Wakelin (1942) to have 50 per cent toxicity at 1 040 μ M

† Non vesicant

A few comments are necessary upon the method of dissolving some of the substances and upon their behaviour

Dichlorodiethylsulphone (HO2)

Table II gives values for the last four experiments and for a previous one considered to be reliable Several earlier experiments were excluded after discovery that solution was apt to be incomplete at a concentration of 1 mg/ml Ringer-phosphate solution owing to the formation of small glass-like globules of the compound which were difficult to see For these purposes the best way was to add c 05 ml Ringer-phosphate to about 30 mg HO₂ in a test tube, warm until the crystals went into solution, and then add up to 60 ml to make 01 ml = $50 \mu g HO_2$ The solution should then be examined for small transparent globules of HO2 and, if these are suspected, the whole warmed As hydrolysis is slow in presence of salt (Peters and Walker, 1923) any slight loss is completely offset by the certainty of complete solution Three separate solutions were used in getting the results of Table II Upon the same solution, results for different brains agreed closely

2 2' 2"-trichlorotriethylamine

Solutions of the pure hydrochloride (kindly supplied by Sir Robert Robinson and Dr Mason) were used any necessary small additions of alkali were previously made to the bottles to compensate for changes in pH produced by the hydrochloride The free base is liberated as an oil at pH.73

Divinylsulphone (DVS)

The experiments quoted were done with a pure specimen from Dr McCombie, supplied through Dr Fell A few mg stirred with appropriate volumes of water (c 5 0 ml) appeared to give satisfactory solution

Phenyl-2-chloroethylsulphone

This compound (supplied by Porton) was dissolved as far as possible by warming It does not hydrolyse to any extent

Iodoacetic acid and iodoacetamide

It was noted that the poisoning induced by iodoacetamide in the first 30 min is relatively greater than that with other compounds, showing that the condensation is very rapid

Thiodiglycol and dihydroxydiethylsulphone

In this experiment the values given for the O₂ uptake are those of the bottles containing pyruvate and pyruvate + substances added, without subtraction of the residuals. These substances had a depressant effect on the residual respiration whereas they will be seen to have none on the values in presence of pyruvate. This is interpreted to mean that there is competition for the active centres in the brei between the last traces of pyruvate present in the residue and these substances.

In order to get a better picture of the accuracy of these comparisons, the behaviour of HO, divinvisulphone, and trichlorotriethylamine was examined more closely Dr R B Fisher, of this Department, kindly tested the figures for HO, and trichlorotriethylamine (Table II) statistically upon the assumption that the relation between percentage inhibitions and log concentrations is linear This was more true for HO, than for trichlorotriethylamine, owing to the hydrolysis of the latter during the 2 hr of experiment The analysis showed a rather wide variance for the figures as the probable value for the ratio between means at the point of 50 per cent toxicity was 43, with a 1/20 chance of the ratio lying outside the limits 26 to 73

In view of this which was due mainly to the difficulty of standardizing the initial rate of action of the poison in the brei, we carried out a few further experiments, making a direct comparison at the molarities required to give approximately 50 per cent inhibition in order to get more information upon the accuracy of this type of experiment Experiments were made with 16 bottles as

TABLE III

COMPARISON OF TOXIC EFFECTS UPON THE PYRUVATE ONIDASE SYSTEM OF THE SAME PIGEON BRAIN BREI TISSUE RESPECTIVELY OF (a) HO $_2$ and trichlorotriethylamine and (b) HO $_2$ and divinylsulphone (DVS) substrate Na pyruvate 0.018M residuals subtracted

(a)	Conc	M 10 4		ge resp μl /g /hr	% inh	ibition
Exp	но.	Trichloro- triethyl- amine	0-30 min	30–120 min	030 min	30–120 min
1970		· —	1980	1849	_	
	09	·	1831	901	75	- 54 8
1971	_		1636	1378		l
	09		1728	766		44 4
		3 87	1311	727	-195	-47 2
1972	↓ —	1	1636	1542		
	0.9		1339	645	18	- 58 2
		3 87	1214	712	26	- 53 9

(b) HO2 and DVS direct comparison no controls with pyruvate alone triplicate estimations HO2 0.91 \times 10 4M , DVS, 0.74 \times 10 4M 52 μg and 26 μg per 3 ML respectively

Pyru- vate	но	DVS			Dıffer	ences
 	+	<i>-</i> -	1263 1296 2204 2115	521 539 902 985	941 819	381 446

follows two 0 (residual), two + pyruvate, three + HO₂, three + trichlorotriethylamine, three pyruvate + HO₂, and three pyruvate + trichlorotriethylamine The results are given in Table III

The statistical estimate made the probable value for the ratio 443, with limits of 272-7.21 (1/20 chance) in agreement with the estimate in Table II This gives the possible limits of accuracy of this type of experiment

Table III (b) confirmed the previous figures On a molar basis divinylsulphone was the most toxic substance of the series

Since the first object of this work was a comparison of toxicity to the pyruvate system with vesication, the values for enzyme inhibition (50 per cent toxicity) were compared with the Porton data for vesication (upon a weight basis) In Table IV HO₂ is put as 100 and some correspondence between vesicant action and toxicity will be

TABLE IV COMPARISON OF 'OXIDASE" TOXICITY AND VESICANT ACTION, $HO_2=100$

Substance	Oxidase toxicity	Vesicant action
Iodoacetamide Divinylsulphone (DVS) Dichlorodiethylsulphone (HO ₂) Iodoacetic acid Trichlorotriethylamine (hydrochloride) Phenyl-2-chloroethylsulphone Dichlorodiethylsulphoxide Thiodiglycol and dihydroxydiethylsulphone	186 198 100 37 19 8 9 nil	Unknown* 2 5 100 Vesicant 10-20 ntl nil

^{*} One of us (R.W) produced on the left forearm a large crythematous patch 8×8 cm and vesicle 3.5×6.0 cm with an application of 1.5 mg iodoacetamide in ethanol

The only marked discrepancy was in the values for divinylsulphone, this is known to be highly toxic to animals by injection, and on the arm of one of us (RAP) small amounts readily We are inclined to consider the formed vesicles available Porton estimate too low, though could not press this in the absence of further data So far as the hypothesis of an attack upon the -SH group in the enzyme is concerned, the data given so far are in agreement, HO2 and divinylsulphone have a µM toxicity (ET50) of 90 and 75 respectively, which is to be compared with the trivalent arsenical toxicity (Peters, Sinclair, and Thompson, 1946) of 17-30 μM , where two -SH groups are attacked per mol The case is different for mustard gas now to be considered

II WATER INSOLUBLE SUBSTANCES Mustard gas (H)

Some early experiments were done by the method used by Berenblum *et al* (1936), in which the substance was mixed with the tumour tissue thoroughly

TABLE V

Concentrations* for immiscible substances and thiodiglycol giving approx 50 per cent decrease in O_2 uptake of pyruvate oxidase system in pigeon brain brei

Substrate Na pyruvate, 0 018M No of experiments in parentheses

Substance	Conc for -50%(µM	Conc for $-10\%(\mu M)$
2 2'-Dı(β-chloroethylthio)-di- ethyl ether Mustard gas Phenyl-2-chloroethylsulphide Thiodiglycol Butyl chloride (tertiary) Butanol (tertiary)	330 (3) 770 (3) 1000 (3)	150 (2) 200 (3) 240 (1)

^{*} Concentrations in this series of experiments only are reckoned in terms of the amount of test substance per 100 mg tissue used

before it was added to the respiration bottles Table V gives some results so obtained

It will be seen that although H is toxic it is—much less so than the arsenicals and divinyl-sulphone. Thinking that this might be due to the inefficient method of introduction of the poison, we tried other—solvents—Table VI gives the results of the early and some later experiments using isopropanol and ethyl and methyl cellosolve

It was possible to use these solvents with a correction, and in fact this was done later in the experiments in this laboratory upon BAL (Stocken, Thompson, and Whittaker, 1947), but it was then thought advisable to find some other method, and the use of lecithin was explored Commercial lecithin (BDH) cannot be used for the emulsification as it is toxic in itself Egg lecithin was therefore prepared as follows (we are indebted to Professor J B Leathes for the details of this preparation)

Preparation and use of lecithin—The yolks of 6 eggs were stirred with 3 volumes of acetone and the solution filtered, the solid residue was stirred with a further 3 volumes of acetone—The residue was then shaken with

TABLE VI

TOXIC EFFECT OF SMALL CONCENTRATIONS OF SOLVENTS UPON PYRUVATE OXIDASE SYSTEM AND THE COMBINED EFFECT OF SOLVENT AND MUSTARD GAS BREI AND DISPERSIONS, PIGEON BRAIN SUBSTRATE Na PYRUVATE, 0.018M (Brei), 0.011M (Dispersion)

Me cs = Methyl cellosolve, 0 025 ml/3 ml Et cs = Ethyl cellosolve H = Mustard gas L = Lecithin

Exp	Substrate	Qo <u>±</u> 0–30 min	Averate rate 30–120 min	Decrease	Dispersion (D) or brei (B)
2212	Pyr alone Pyr + <i>iso</i> propanol 0 84% Same + H	13 05 10 78 9 90	9 98 8 61 6 21	1 37 3 77	В
2223	Pyr alone Pyr + isopropanol	19 46 14 08	11 58 9 22	2 36	В
2224	Pyr alone Pyr + isopropanol	16 36 14 30	11 43 9 56	1 87	В
2226	Pyr alone Pyr + propanol	16 90 13 50	11 57 9 00	2 57	В
2227	Pyr alone Pyr + isopropanol Pyr + isopropanol + 800 µg H	17 01 12 96 11 37	11 34 9 51 5 88	1 83 5 46	В
2323	Pyr alone Pyr + Me cs	15 53 6 21		9 32	D (dialysed)
2327	Pyr alone Pyr - Et cs Pyr + Et cs - 800 µg H Pyr - L Pyr - L + H	9 59 7 52 5 48 10 30 8 13		2 07 4 11 2 17	D (dialysed) (extra for H -2 03)

acetone for 2 hr in a mechanical shaker, the suspension filtered, and the residue suspended in absolute ethanol overnight. After filtration the residue was again suspended in absolute ethanol and shaken mechanically for 2 hr. The ethanolic filtrates were combined and concentrated in vacuo at $38-40^{\circ}$ to a small volume and made up so that 1 ml = 100 mg. When this solution is kept in the ice chest, a deposit of sphingomyelin gradually forms. We have found the product from this relatively crude ethanolic solution quite suitable for our purpose, and it gives no inhibition of respiration with a brei

For experiment, weighed amounts of H (c 8 mg) were added to 50 mg lecithin (after removal of ethanol *in vacuo* in a small test tube) and an intimate mixture was made with a glass rod. Immediately before being added to the respiration bottles, the emulsion was treated with 0.5 ml. Ringer-phosphate solution with vigorous stirring, followed by a further 1.5 ml. to a volume of 2.0 ml. From this a usual addition to the bottles was 0.2 ml. (5 mg. lecithin)

Experiment 2254 (Table VII) shows that the amount of lecithin added to the 30 ml fluid in the respirometer bottles can be varied from 1-5 mg without change, but that 10 mg may reduce the effect of the H In this experiment the H $(50 \mu g)$ was potentiated by addition of diethanol dithiocarbamate (Peters and Wakelin, 1947) (1 mg per bottle)

TABLE VII

exp 2254 inhibitory effect of potentiated H (420 μM) upon Qo2 of pyruvate oxidase system of pigeon brain brei with varying amounts of lecithin present Na diethanol dithiocarbamate (1 9 mM) also present

Substrate Na pyruvate, 0 018M

Lecithin	Qo ₂	%
+ 1 mg	-4 35	-59
+ 5 mg	-4 62	-62
+ 10 mg	- 3 67	-46

We have employed this method of adding oily substances extensively and have confidence in the results obtained. The arguments in favour of the use of lecithin may be summarized.

- (a) Lecithin is a normal constituent of brain and other tissue, its addition therefore only increases slightly the amount already present in the tissue
- (b) When properly purified and added alone, it has no effect upon the Qo₂
- (c) It has no competitive action with H (Holiday, Ogston, Philpot, and Stocken, 1940, Ogston et al 1948) but merely reduces the rate of hydrolysis, as has been proved experimentally by Ogston (personal communication)
- (d) There is no increased toxicity if the combined emulsion of lecithin and H is allowed to

stand before addition of the Ringer-phosphate, or even if it is warmed to 38°, hence it does not form a toxic addition compound Experiment 2304 shows this (Table VIII)

TABLE VIII

EXP 2304 BRAIN BREI (PIGEON) QO₃ FOR PERIOD 35–120 MIN SUBSTRATE Na PYRUVATE (0 018M), LECITHIN IN ALL BOTTLES

Additions	Qo ₂
No addition 800 µg H*	10 45 5 89
800 µg H stood for 1 hr at room temperature after thorough mixing* 800 µg H warmed in bath for 20 min after	6 18
thorough mixing*	5 97

* The Ringer phosphate was added to the thoroughly stirred lecithin immediately before addition to the bottles

Tables IX, X, and XI give a selection of results obtained for mustard gas with dispersion, with brei, and with slices, these are given separately because there are differences in the detail of the effect. The toxicity (ET50) works out at more than 1,000 μM . This is clearly a relatively low toxicity compared with those of divinylsulphone and the arsenicals, even if we allow for a slight loss by preliminary hydrolysis before the poison has time to act, it is of the same order of toxicity as that of iodoacetic acid to this enzyme

Dispersion—The action upon pyruvate respiration (Table IX) is noticeable in the first 10 min period of observation and fully established after 20 min, the maximum effect was seen in the respiration period 10–20 min. Only 3 out of 15 experiments showed significant inhibition in the residual respiration. The protocol of 2 typical experiments out of the large number performed are given.

TABLE IX

dispersion (pigeon brain) change in rate of respiration in μl /g /hr O_2 uptake, due to addition of lecithin + H (800 μg H in 5 mg lecithin per bottle) substrate pyruvate respiration period in min

Exp No	0–10	10–20	20–30	30–40	Average	% change
2047 2061	-660 -225		-580 -835		599 560	-18 -16

The values given represent the decreased rate of O_2 uptake over the periods mentioned, i.e., that for pyruvate minus pyruvate + H for the reason why this procedure is used see Peters (1938)

Brei —Fig 1 shows the rate of poisoning of brei in a typical experiment. Table X gives the percentage inhibitions observed in a series of experiments with different concentrations of H lowest amount producing a noticeable change is $100 \mu g H/3 ml$, the effect depended much upon the efficiency of emulsification and the rapidity with which the emulsion was added to the bottles The effect was noticeable in the first 15 min but did not become maximal until the 30-60 min Experiment 1993 shows that, if first period allowed to hydrolyse in an aqueous solution, the H produced no action, as is readily understood from consideration of the kinetics (Ogston et al., 1948)

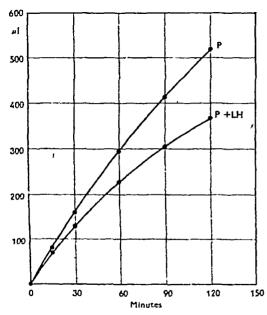


Fig 1—The effect of H in lecithin (LH) upon the respiration of brain brei with sodium pyruvate (P) as substrate Ordinate ml/g/hr Abscissae min Pigeon brain brei

In the final experiments, the changes produced by addition of 800 μg of H to each bottle (167 mM) were of the order of -16 per cent for period 0-30 min and -30 per cent for 30-12 min -1e, the concentration required to produce a 50 per cent change was greater than 1,000 μM

Shees—Exp 2065 (Table XI) confirms that in slices too lecithin has no influence on the respiration, H here inhibited the residual respiration. In Exp 2068 it will be seen that with glucose the poisoning effect is increasing up to 3 hr. Exp 2072 was a comparison between the action of H with pyruvate and glucose as substrates, in which it will be noticed again that poisoning of the respiration in presence of glucose is increasing up to 3 hr.

TABLE X

Brei (Pigeon Brain) — Changes in rate of respiration Given in Per Cent, due to addition of H in Lecithin in various concentrations—substrate—Na pyruvate, 0 018M (residuals subtracted)

Evn			change		
Exp	per bottle (μg)	0–30 min	30–120 min		
1987 1988 1990 1991 1992 1994	750 650 325 750 750 515 1180 590 400	-45 +5 +11 -223 -16 -126 -20 +1 -342	-50 1 -19 0 -29 8 -43 5 -23 5 -41 7 -23 5 -45 2		
1997 1998 1999 2000 2003* 2004* 2006*	660 330 510 900 760 360 120 50 30	10 7 +3626 524 37 54412 327 8	-27 5 -9 4 -30 7 -47 8 -47 -23 4 -6 5 -17 5 -12		

^{*} Residuals not subtracted

H-lecithin added after hydrolysis in Ringer-phosphate for 30 min at 38°

1993	1080	-82	0

Note In most recent experiments, as the result of further emulsification and quicker working, the changes produced by $800 \,\mu g$ H/bottle ($1.67 \times 10^{-3} M$) were of the order of -16 per cent for 0-30 min, and -30 per cent for 30-120 min

DISCUSSION

In a comparison of the rates of poisoning and of hydrolysis for H it is to be noted that in accord with the theory of the kinetics of replacement of chlorine in H (Ogston et al., 1948) any substitution with a group in the enzyme must take place during the hydrolysis, most of this occurs in these experiments in the first 15 min and is complete in 30 min. The rate of action upon the dispersion was consistent with this, that upon brei and slices was too slow One conceivable explanation for this difference was conversion of H to an oxidation product, in a direct test of this we could find no increased O, uptake owing to the addition of H which might suggest oxidation to sulphoxide It seems more likely that there is adsorption into mactive lipoid parts of the brei or slice with slower penetration to the active centres, this could explain most of the effect in slices when pyruvate

TABLE XI

SLICES (PIGEON CEREBRAL HEMISPHERES) CHANGES IN RATE OF RESPIRATION DUE TO ACTION OF H IN LECTHIN (L) $(800 \mu g/ml)$

Exp	Substrates	Respiration rate in μ l /g /hr during periods (min)						
ΣνÞ	Duositates	0-15	15-30	30-60	60-90	90-120	120-150	150-180
2065	None (a) L (b) L + H (c) (b)-(c)	1851 1846 1670 —176	1585 1555 1406 —149	1290 1240 1055 —185	976 967 689 278	809 761 471 —290	698 604 307 —297	-
2068	Gi only (d) Gl + L (e) Gl + L + H (f) (e)-(f)		3350	3255 3320 2875 445	3255 3135 2630 505	3247 3275 2465 —810	3147 2990 2155 —835	3057 3000 1995 —1005
2072	Pyr + L (g) Pyr + L + H (h) (g)-(h)	3625	4260 3485 775	3794 2997 —797	3006 2362 646	2910 2055 855	2630 1745 -885	2320 1341 -979
	$GI + L(J)$ $GI + L + H(\lambda)$ $(J)-(\lambda)$	3720 3085 -640	3785 3070 -715	3650 2514 —836	3445 2440 -1005	3500 2400 —1100	3340 2120 -1220	3265 1915 1915 -1350

is the substrate, it would leave unexplained the gradual increase in poisoning observed with slices in glucose solutions. Since this work was reported in 1940, Dixon and Needham and colleagues (for review see 1946) have produced much evidence in support of the poisoning of hexokinase by H which would explain the latter effects better. Other factors contributing to the slower action may be the adsorption and half product formation (Peters and Wakelin, 1947) and also perhaps sulphonium salt formation (Stahmann et al. 1946)

III SELECTIVE ACTION OF H

In 1936 (Peters) it was shown that HO₂ poisoned the lactate oxidase system of brei selectively at the pyruvate stage, since Keilin's cytochrome system is common to the oxidation of both lactic acid and pyruvic acid, this experiment indicated that the cytochrome system and the lactate dehydrogenase were much less sensitive to HO₂ than pyruvate dehydrogenase, it also excluded poisoning of cozymase. Though the sulphone combined with glutathione, the latter did not reactivate the poisoned system, except for the more powerful action of HO₂, the effect resembled that of iodoacetate (Peters, Rydin, and Thompson, 1935)

After showing that H could poison the pyruvate oxidase system we thought it advisable to confirm the earlier conclusions for HO_2 by direct experiment and to extend them to H. Direct experiments were therefore done (1) upon the vitamin B_1 component, (2) upon the cytochrome system, (3) upon

the total succinate oxidase system in brain, and (4) upon amino-acid oxidase, especially because this contained the adenine flavine dinucleotide as a component

Aerobic experiments

Cocarboxylase—It had been shown previously that addition of vitamin B₁ did not stop the poisoning effect of HO₂. In Exp 1875 (Table XII) it was shown that addition of large amounts of cocarboxylase, now known to be the active component (Banga, Ochoa, and Peters, 1939), does not restore the poisoned respiration

Cytochrome system - Cytochrome oxidase and cytochrome C were prepared by the method of

TABLE XII

SEPT 6, 1939 FAILURE OF THE ADDITION OF COCARBOXYLASE TO RESTORE ACTIVITY OF PYRUVATE OXIDASE SYSTEM POISONED BY DICHLORODIETHYLSULPHONE (HO.) PIGEON BRAIN DISPERSION, SUBSTRATE PYRUVATE, 0 018M

ΗΟ .	Cocarboxylase	O ₂ uptake	%
μΜ	µg	μl /g /hr (20 mm)	change
100 100	— 18	2645 2242 2242	-15% -15%

 $0.2~\mu g$ cocarboxylase should produce a maximum effect under these conditions in a vitamin B_1 deficient brain

Keilin and Hartree (1938), and hydroquinone was used as substrate Table XIII gives the results

Arsenite, HO₂, and divinylsulphone, all of them vesicant or potentially vesicant substances, produced no effect, 10 µg As Os poisoned the pyruvate system to the extent of 85 per cent and 25 µg divinvisulphone, 50 per cent H (emulsified in lecithin) showed inhibitions up to 7 per cent, when cytochrome C was present in maximum concentration, and up to 20 per cent when the latter was present in submaximal amounts That this effect was mainly on the cytochrome C component was suggested by two other experiments in one (Exp 6) the action upon the cytochrome oxidase, to which cytochrome C was added before mustard gas-lecithin, was compared with the effect when cytochrome C was added after incubation for 15 min with H, by this time much of the H would have been hydrolysed or rendered ineffective by combination with phosphate, etc., in another

TABLE XIII

EFFECT OF MUSTARD GAS, SOME SULPHONES, AND ARSENITE UPON KEILIN'S "CYTOCHROME SYSTEM"

All bottles contained cytochrome oxidase (0 3 ml), cytochrome C (0 7 per cent solution as stated), 5 mg hydroquinone in 0 2 ml (1 5 \times 10-2M) was added after equilibration L=Lecithin H=Mustard gas HO₂= Dichlorodiethylsulphone DVS = Divinylsulphone

		· · · · · · · · · · · · · · · · · · ·			
Ьлр	Cyt (ml)	Additions	Time in min	μl O ₃	% change
1 24 4 40	0 02 0 02 0 02 0 02	None + L (7 5 mg) + L + H (1,200 μg) + HO ₁ (200 μg)	20 20 20 20 20	224 243 250 248*	-178
25 4 40	0 2 0 2	None +As ₃ O ₃ (200 μg)	30 30	262 265	41 5
3 7 8 40	0 0 0 0	\one + L + L + H (1 200 \(mu\text{g}\)) + L + D\(S\) (150 \(mu\text{g}\))	20 20 20 20 20	344 336 286 334	-15 -
- 4 - 8 40	0 0 0 02 0 02	+ L (5 mg) + L + H (800 μg) + L (5 mg) + L + H (800 μg)	20 20 20 20 20	325 202 353 328	-11 -7
5 5 8 40	0 0 0 1 0 1	-1 - H (800 µg)	15 15 15 15	249 207 351 343	-20† -2‡
6 + 9 40	0 1 0 1 0 1 0 1	$\begin{vmatrix} +1 \\ +1 - H(800 n_{r}) \\ +1 \\ +1 + H(800 n_{g}) \end{vmatrix}$	15 15 15 15	405 386 420 383	- 5 - 74

Note Vessels containing H had 0 05 ml N/10 NaOH per 800 µg to neutralize the acid formed from H

(Exp 7) cytochrome oxidase was incubated for 15 min with and without lecithin, the residue centrifuged and tested with cytochrome C. There was no essential difference from the experiment carried out in the presence of the poison, where much cytochrome C was present the effect was reduced

EXP 7 Equal amounts of cytochrome oxidase suspension were incubated (A) with lecithin only and (B) with H in lecithin for 20 min, N/10 NaOH was added at 10 min to B to adjust the pH After being centrifuged, aliquot amounts were compared for activity with and without cytochrome C (0 1 ml)

	Cyto C	μl O ₂	% change
A B A	0 0 +	292 259 363	-13
В	+ :	341	-65

These inhibitions of cytochrome C indicate that H can combine with it, they are of no significance in the experiments upon brain brei as the cytochrome C is not a limiting factor, this was tested directly in one experiment 8 where there was no significant change

Exp 8 Brain dispersion from the cerebrums of two pigeons was treated with lecithin (L) and H (800 μ g) in lecithin as usual, after 10 min preliminary incubation period and a further period of 5 min , 0 15 ml cytochrome oxidase (cyto ox) + 0 05 ml cytochrome C (cyto C) were added from a dangling tube to see whether this improved respiration Substrate Na pyruvate, 0 023 M Average of duplicates

Addition	μl O _z /g /hr 30 min	Change
L only HL L+cyto ox +cyto C HL+cyto ox +cyto C	2933 2622 2780 2452	-311 -328

Succinate oxidase system — Table XIV is a summary of tests made upon the relative toxicity of these compounds to succinate and pyruvate respiration in the same brei. In each test there is 2-3 times larger inhibition of the pyruvate than that of the succinate respiration, the effect is therefore selective. There is a sporadic slight poisoning of the succinoxidase system, not yet explained, which is connected with the state of the tissue, because in ground muscle preparations the succinodehydrogenase is not poisoned by H

Exp 2027 is a striking variation of the same theme. We have shown separately (Peters and

TABLE XIV

COMPARISON OF TOXICITY TO PYRUVATE AND SUCCINATE SYSTEMS SUMMARY OF CHANGES (INHIBITIONS) DUE TO POISON IN $\mu l/g/hr$ for Period (2) 30–120 min

Poison*	Ехр	Resi- dual	Pyru- vate	Suc- cinate
HO (0 173 mM) H (as oil) (0 592 mM) H (in lecithin) (1 31-	1893 1932	221 167	- 1120 - 696	-282 -273
1 72 mM) "" "" "" H (0 315 mM) in leci-	2032 2099 2034 2036	-92 -251 -120 -100	-631 -698 -	-264 -139 -125 -341
thin and diethyldi- thiocarbamate (1 56 mM)	2027	-346	—1136	0

* Per 100 mg. tissue

Wakelin, 1943, 1947) that diethanoldithiocarbamate potentiates the toxicity of H. Here an amount of H, which would have had no action upon pyruvate oxidation by itself, had a large effect in presence of diethanoldithiocarbamate, under the same conditions succinate oxidation was unaffected. Since the cytochrome system is common to both oxidations, we have a remarkably clear selective inhibition.

Anuno-acid oxidase—This was prepared from pig's kidney by the method of Krebs (1935). The system employed for study consisted of 20 ml enzyme extract and 1 ml buffer (pH 73) with or without additions made up in buffer solution. The enzyme extract was made by shaking 1 g dry powder with 40 ml water at room temperature for 10 min and then centrifuging. Table XV gives the results and shows that none of the poisons tested interfered with the amino-acid oxidase.

TABLE XV

EFFECT OF SOME POISONS UPON THE AMINO-ACID OXIDASE
FROM LIDNEY

	O ₂ uptake (µl) in 55 min		
No addition dl-Alanine (0	22M) ,,	+ 5 mg lecithin + 700 µg H in lecithin + 200 µg iodoace- tic acid + 200 µg dichloro- diethylsulphone	25 382 394 394 382 367

system, they cannot therefore mactivate the adenine flavine dinucleotide component

The experiments in this section indicate that the pyruvate oxidase system is selectively poisoned by H at concentrations which do not affect the other parts of the system, though the concentration of H required for this toxic effect is much larger than that of arsenicals and the two relevant sulphones. Since this work was reported Dixon and Needham (1941) have shown that H is toxic to the pyruvate system of B coli, an enzyme obtained by Still (1940) in clear cell-free solutions, the optimal pH for their enzyme was pH 6.2, differing from that of the brain experiments, pH 7.3

Dehydrogenase experiments -- So far the experiments described have been upon partly organized tissue systems, which is reasonable when the effect upon the living cell is being especially considered But it was desirable to know how the isolated dehydrogenases behaved Many experiments were therefore done upon the actions of several of these compounds upon standard dehydrogenase preparations, only representative experiments are given in Table XVI for the 3 substances H, HO₂, and divinylsulphone In muscle preparations H had no appreciable effect upon succinate, malate, or lactate dehydrogenase, with brain tissue H had a slight and variable effect on succinodehydrogenase, but the action of H upon pyruvate dehydrogenase was always greater Nevertheless, as with the arsenicals, the action of H upon the pyruvate dehydrogenase is less than upon the total oxidase, suggesting that there is a factor in the aerobic system, perhaps the phosphokinase (Dixon and Needham, 1946), which is more sensitive

The toxic action of HO₂ and of divinylsulphone was greater upon the pyruvate dehydrogenase than upon the other dehydrogenases, on the former the effect of comparatively low concentrations was considerable, which is in agreement with the investigation upon the total oxidase

DISCUSSION

HO₂, divinylsulphone, and H show a selective action upon the component of the pyruvate system dealing specifically with pyruvate, after completion of these experiments R van Heyningen (1941) and workers in the US considerably extended the number of enzymes tried, very few were sensitive to H, hexokinase (Dixon and Needham, 1946, van Heyningen, 1941) being an outstanding exception Like the arsenicals, HO₂ and divinylsulphone appear to be combining with an -SH group, this is consistent not only with the toxicity values but

TABLE XVI

effect of dichlorodiethylsulphone (HO2), divinylsulphone (DVS), and mustard gas (H) upon pyruvate and some other dehydrogenases pyruvate, 0.018M, Na succinate, 0.082M, malate, 0.0585M, lactate, 0.069M, both the latter had cozymase added, total volume $1.5~\mathrm{mL}$

Exp	Source and type	Incubation time (min) for poison	Substrate	Decolorization time (in min) for methylene blue				
				Substrate alone	HO ₂ 100 μg 200 μg		DVS 25 μg	
1	Brain	40	{ Pyruvate { Succinate	15 13½	40 24	140 7 22		
2	Brain	40	∫ Pyruvate	16 6½	37½ - 9	70 10		
3	Brain	40	∫ Pyruvate Succinate	23 15	35 17		40 17 1	
4	Brain	40	Pyruvate	12 1	i —		$a (100 \mu g)$	
4 5	Brain	40	Pyruvate	17			$80 (50 \mu g)$	
6	Muscle	0	Malate	10	_	10+	-	
_	1	0	Succinate	19	<u> </u>		19 (240 μg)	
7	Muscle	40	Malate	71/2	_		71/2	
					H (800 μg)			
8	Brain	20	{ Pyruvate { Succinate	13 1 11 1	22½ 12½	_	$O_2 = 0.70 \text{ m}M$	
9	Brain	40	Pyruvate Succinate	28 28	42 30	$25 \mu \text{g} \text{DVS} = 0 141 \text{m}M$		
10	Muscle	20	Succinate	91	9 1	$800 \mu g H = 3 28 mM$ emulsified with lecithin		
11	Muscle	15	∫ Malate ∫ Succinate	50 20 4 9	50 22			
1,2	Muscle	40	Malate		4 9 1	Cinaismoa W	im worthin	

also with the earlier work (Peters and Walker, 1924-5) on the abolition of -SH reactions in skin slices, etc, by some of these substances, and with other facts known now about the action of the sulphones (Ford-Moore and Lidstone, 1940, Banks, Boursnell, Francis, Hopwood, and Wormall, 1946, Stahmann, Golumbic, Stein, and Fruton, 1946), on the other hand, the idea that H combines as well with the -SH groups in the proteins as it does with thiol groups in compounds like glutathione must be abandoned In the earlier work (1924-5) it had been found that H, unlike its sulphone when free from dithio compounds, did not abolish the nitroprusside reaction of the fixed -SH groups in tissues, this was set aside at the start of the war, erroneously as it turned out Other workers (van Heyningen, 1941 Bailey and Webb, 1944, Banks 1946) as well as ourselves (Peters and Wakelin, 1947) have concluded that in proteins the attack of H is only partly upon the -SH groups and that it combines with other groups like COOH In view of this, it cannot yet be decided how H attacks the pyruvate oxidase system In another communication (Peters and Wakelin, 1947) the suggestion was made that potentiation by diethvl dithiocarbamate directs the attack of H to the -SH

groups In this relation, with work upon lacrimators, vesicants, and -SH groups, the work of Z Bacq and his colleagues (for summary see Z Bacq, 1946) is of interest

In regard to the toxicity of HO_2 and of the arsenicals to the pyruvate oxidase system, it is interesting to note that Fell and Allsopp (1939, 1946) have found for the lethal doses to tissue culture preparations, 11-18 μM for lewisite oxide and 75 μM for HO_2 , these amounts are surprisingly close to those which inactivate this enzyme. If it is not the pyruvate system which is attacked, it must be some as yet unknown enzyme of equal sensitivity

Note Since completion of this report, it has been concluded that the pyruvate oxidase system as studied here includes the tricarboxylic acid cycle (Coxon, Liébecq, and Peters, see Peters, Dixon lecture, 1948)

SUMMARY

- 1 The toxicity of several non-arsenical vesicants and related substances to the pyruvate oxidase system in pigeon brain has been determined
- 2 The most toxic substances were dichlorodiethylsulphone (HO₂) and divinylsulphone it is

suggested that these compounds attack an -SH group

- 3 Mustard gas (H) was less toxic, but with the help of emulsification with lecithin it could serve as a test for antidotes. There is evidence that it is not attacking the -SH group alone
- 4 All three substances attacked the pyruvate system selectively, and the pyruvate components of the total oxidase system, the toxic action upon succinic dehydrogenase, malate dehydrogenase, and lactate dehydrogenase was much less marked. and no toxic action upon amino-acid oxidase was observed with the concentrations used

We wish to acknowledge the help of members of the team during this work, which was carried out during the war for the Ministry of Supply We are grateful to the Chief Scientific Officer for permission to publish, to Dr Ing and Mr Philpot for diethanoldithiocarbamate, and to Prof Keilin for preparations of cytochrome C and succinodehydrogenase

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THE VASODEPRESSOR ACTION OF NORADRENALINE

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In their classical study of the relation between chemical structure of amines and their sympathomimetic actions. Barger and Dale (1910) noted that a dose of ergotoxine sufficient to reverse the pressor effect of dl-adrenaline in the spinal cat did not reverse that of dl-noradrenaline pressor response of the latter substance was greater than that of the former, but relaxation of the isolated non-pregnant uterus was slight Since that time many workers (Tainter, 1931, Greer, Pinkston Baxter, and Brannon, 1937, 1938, Gaddum and Goodwin, 1947) have confirmed these findings, and found that vohimbine and most ergot alkaloids in addition to ergotoxine depress but do not reverse the pressor response to noradrenaline Naturally occurring *l*-adrenaline and synthetic dl-noradrenaline were used for purposes of comparison On some occasions, however, the action of noradrenaline was wholly inhibited or even slightly reversed by ergotamine. though never to the same extent as that of adrenaline (Stehle and Ellsworth, 1937) (1937) showed that certain dioxane derivatives such as 933F and 883F failed to reverse the pressor action of noradrenaline but did reverse that of adrenaline Dibenamine (Nickerson and Goodman 1947) has also been used as an adrenaline antagonist

The difference in response of similar doses of the two amines on the blood pressure of cats and dogs after the administration of an antagonist has been used (a) to support the theory that the substance liberated on stimulation of the hepatic nerves is noradrenaline or some similar substance (for references see West, 1947b), and (b) to confirm the observation that extracts of various mammalian organs (except placenta) contain a pressor substance with properties like those of noradrenaline (Euler, 1946) It has now been possible to demonstrate the vasodepressor action of noradrenaline in the cat by using large doses The successful resolution of arterenol (dlnoradrenaline) by Tainter, Tullar, and Luduena (1948) has enabled this work to be completed with both dl- and l-noradrenaline

METHODS

In different experiments, spinal cats, cats anaesthetized with chloralose or urethane, and rabbits anaesthetized with urethane were used Blood pressure records were taken from the carotid artery, injections of the drugs were made into the femoral, jugular, or splenic veins, or into the external iliac artery so that the injected solution passed into the vessels of the opposite leg In some cats, injections were also made into one of the two main splenic arteries Movements of the duodenum and uterus were recorded directly The adrenaline antagonists used were ergotoxine (5 mg/ kg), ergotamine tartrate (2 mg/kg), and dibenamine For enhancement of the responses (15 mg/kg)cocaine hydrochloride (8 mg/kg) was given intravenously

In one experiment, samples of heparinized blood were taken from the femoral artery, and after being rapidly cooled were subjected to biological analyses (West, 1947b) Dialysed samples were tested on the perfused blood vessels of the frog (West, 1947a), whi'st undialysed plasma samples were tested on the isolated uterus and ileum of the rat (de Jalon, Bayo, and de Jalon, 1945) Solutions of *l*-adrenaline, *dl*- and *l*-noradrenaline were prepared from the pure substances, which were kindly supplied by Dr M L Tainter

RESULTS

During the comparison of the pressor actions of adrenaline and dl-noradrenaline in the cat, it was confirmed that ergotoxine and ergotamine antagonized the vasopressor action of adrenaline so that the vasodilator component was unmasked No typical reversal occurred with noradrenaline since it has little vasodilator activity in comparable doses When, however, the dose of noradrenaline was considerably increased (to more than twenty times the corresponding reversal dose of adrenaline), the vasodepressor action of noradrenaline became apparent (Fig. 1) double vagotomy, the fall of blood pressure following the injection of 250 µg noradrenaline into the femoral vein was still present venous atropine likewise was without effect on this response It is worthy of note that the fall of blood pressure was prolonged, in contrast to

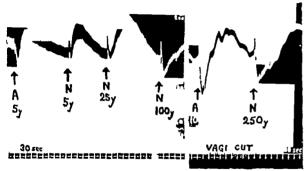


Fig 1—Cat 2 5 kg Chloralose Ergotoxine The effect on the blood pressure of doses of *l*-adrenaline (A) and *dl*-noradrenaline (N) before and after vagotomy All injections into the femoral vein

the large transient fall after the administration of adrenaline, and never reached the maximum depression shown by doses of adrenaline. These two facts suggest a definite difference in the mode of production of the vasodepressor action

In cats under chloralose receiving cocaine and ergotamine intravenously, similar reversals occurred, and in addition relatively small doses of noradrenaline produced falls of blood pressure. In one animal which had a steady blood pressure of about 200 mm. Hg throughout the whole experiment, $10~\mu g$ of adrenaline by jugular vein produced maximal vasodilatation, whereas

250 μg of noradrenaline exerted the maximal effect for this drug. These results were independent of the anaesthetic, since comparable results were obtained in spinal cats and in cats under urethane and chloralose. In a spinal cat, given cocaine and ergotoxine, the vasodepressor action of large doses of noradrenaline was only shown when the blood pressure had been raised to about 100 mm. Hg. As with the adrenaline vasodilatation, there is a limit to the amount of vasodepression obtainable by these drugs.

In a cat under urethane (Fig. 2), 30 µg dlnoradrenaline after dibenamine produced a fall of blood pressure and relaxation of the ileum in vivo comparable with that produced by 1 ug In this experiment, samples of heparinized blood were taken from the femoral artery before the administration of noradrenaline and at the peak of the fall of blood pressure after its administration Half the number of samples were cooled immediately, centrifuged, and the plasma tested on the isolated uterus and ileum of the rat, the other half were cooled immediately, dialysed against N/100 HCl containing 01 g glycine per 100 ml, and the dialysate tested on the perfused blood vessels of the frog The results of the assays were calculated as noradrenaline and as adrenaline, and indicated that noradrenaline could be detected in the blood in

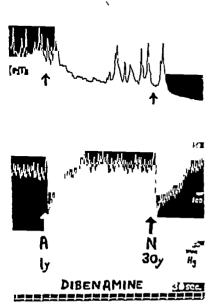


Fig 2—Cat 2.0 kg Urethane Dibenamine Relaxation of the gut and fall in blood pressure produced by 1 μg adrenaline (A) and 30 μg noradrenaline (N) intrafermorally

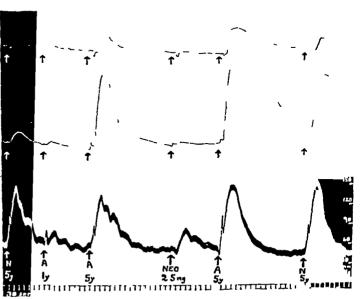


FIG 3—Cat 2.5 kg Chloralose Top record, right nictitating membrane, middle, left denervated nictitating membrane, bottom, blood pressure Potentiation of the /-adrenaline (A) and d/-noradrenaline (N) response by the intravenous injection of 2.5 mg neoantergan (Neo)

a concentration of $10-15~\mu g/ml$ after the intravenous administration of $30~\mu g$ It is possible that a small quantity of noradrenaline may be methylated, and that the subsequent fall of blood pressure may be due to adrenaline, this large dose of noradrenaline, on the other hand, may liberate small amounts of adrenaline from other tissues. The tests on the samples of blood indicated, however, that most of the noradrenaline was present as such, adrenaline (0 23-0 50 $\mu g/ml$) was detected in the blood before the injections of the amines

Effect of antihistamine substances on the vasodepressor response

It is well known that the motor actions of adrenaline are potentiated by antihistamine substances, presumably by antagonism of the histamine liberated during the adrenaline response It seemed of interest, therefore, to determine the effect of antihistamine substances on the noradrenaline response, before and after ergotoxine dibenamine Neoantergan intravenously (1 mg/kg) potentiated both the l-adrenaline and the dl-noradrenaline actions on the blood pressure and nictitating membranes (one denervated) of a cat under chloralose (Fig 3) Benadryl and antistine in suitable doses potentiated these actions in a similar manner After ergotoxine, however, the depressor responses to 5 μ g adrenaline and 100 µg noradrenaline were un-This indicates that the vasodepressor affected response to large doses of noradrenaline after ergotoxine or dibenamine is not due to a liberation of histamine

Reversal of the vasodepressor response

Recently Neil, Redwood, and Schweitzer (1948) reported that the depressor response to aortic or sinus nerve stimulation in cats under pentobarbitone was converted into a rise of blood pressure by the intravenous injection of chloralose The exact mechanism by which this change occurred was not determined amounts of pentobarbitone were therefore administered to cats under chloralose In Fig 4, cocaine and dibenamine were first given intravenously, followed by vagotomy and neoantergan Doses of 1 µg adrenaline and administration 30 μ g noradrenaline produced the typical falls in blood pressure and relaxation of the nonpregnant uterus Pentobarbitone (7 mg) was then given and the cat was left for one hour After this time, the vasodepressor response to large doses of noradrenaline was converted to a rise of blood pressure The normal reversed action of adrenaline was unaffected, as also was the response to small doses of isopropylnoradrenaline (Fig 4, Is) After some time spontaneous reversion of the pressor to the depressor response with noradrenaline occurred In order to exclude the possible effects of altered pulmonary ventilation on the blood pressure, this experiment was repeated with similar results under artificial respiration with bilateral open pneumothorax

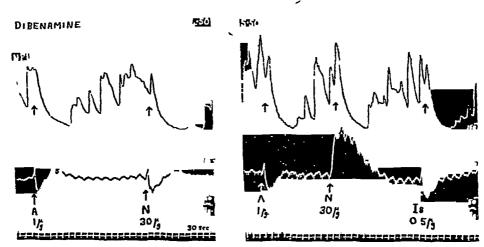
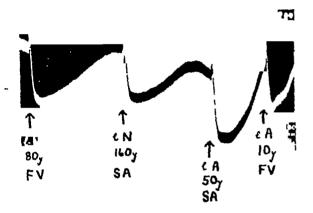


Fig 4—Cat 18 kg Chloralose Dibenamine and cocaine Vagi cut Neoantergan 1 mg/kg intravenously Relaxation of the non-pregnant uterus and fall in blood pressure produced by intrajugular doses of adrenaline (A) and noradrenaline (N) Between the left and right records, a period of one hour during which time 7 mg pentobarbitone were given intravenously Note the reversal of the noradrenaline action on the blood pressure but not that of adrenaline The response to 0.5 µg isopropyl-noradrenaline (Is) was also unaffected

Injections by other routes

The more active l-noradrenaline was used for these experiments. In cats under chloralose, it was confirmed that the ratio of equipressor doses of *l*-noradrenaline and *l*-adrenaline was about 0.6 (Tainter, Tullar, and Luduena, 1948, Graham, After the ergot alkaloids or dibenamine, both substances exhibited depressor responses by the femoral, jugular, and intraportal routes, but the ratio of doses producing maximal vasodepression was reduced from 30 1 (Fig 2) to 8 1 (Fig 5, FV) for the femoral route When injected into the artery supplying the caudal end of the spleen of cats without ergot or dibenamine, noradrenaline produced pure rises of blood pressure at all dose levels, whereas adrenaline injections gave small rises of pressure followed by large falls After dibenamine, similar injections of both drugs produced depressor responses, that of noradrenaline being much less marked than that of



30 sec

Fig 5—Cat 3 2 kg Chloralose Ergotoxine The effect on the blood pressure of doses of *I*-adrenaline (A) and *I*-noradrenaline (N) injected into the splenic artery (SA) and the femoral vein (FV)

adrenaline (Fig 5, SA) These effects were independent of the anaesthetic, since comparable results were obtained in cats under urethane or ether. As reported earlier (West, 1948), the latent period of noradrenaline injections by splenic artery was always longer than that of adrenaline

When injected into the external iliac artery (so that the injected solution passed into the vessels of the opposite leg), 1 μ g adrenaline after cocaine and dibenamine was required to cause the same fall of blood pressure as 0.5 μ g adrenaline by jugular vein. When noradrenaline was injected in a similar manner, 15 μ g by the jugular vein produced a comparable fall of blood pressure, but all intra-arterial doses up to 100 μ g failed to

elicit the true vasodepressor response evidence that the vasodepressor responses of adrenaline and noradrenaline are the result of different mechanisms was shown by perfusion experiments. The hind limbs of cats were perfused with oxygenated Locke's solution at 37° C. and the outflows recorded in a drop-timer (Gaddum and Kwiatkowski, 1938) In this preparation, small doses of adrenaline caused vasodılatatıon after dibenamine (given whereas similar doses of noradrenaline were without effect. In addition, an increase in the dose of the latter had no vasodepressor action

Experiments with rabbits

In a few rabbits, it was shown that the ratio of equi-pressor doses of l-noradrenaline and ladrenaline was about 20, confirming previous observations (West, 1948) that adrenaline is more active than noradrenaline in this animal ergotoxine, pressor responses of both drugs were almost wholly reduced but not reversed Cannon and Lyman (1913) reported that the rabbit, in contrast to the cat, appears to lack a sympathetic depressor component capable of being unmasked by blocking agents such as ergotoxine doses of l-adrenaline and l-noradrenaline (up to 1 mg) failed to elicit any depressor action lar results were found in rabbits after dibenamine, though it was difficult to administer this drug satisfactorily to these animals

DISCUSSION

Previous workers have shown that a dose of a sympatholytic agent sufficient to reverse the pressor effect of adrenaline does not reverse that of noradrenaline Equi-pressor doses of the two amines before the administration of the antagonist were used to demonstrate this difference in response The observation that noradrenaline in suitable doses can produce regular vasodepressor responses in cats which have been treated with ergotoxine or dibenamine is important in blood pressure only occurs after large doses of noradrenaline, also it is always more prolonged than that produced by adrenaline, and never reaches such low levels West (1948) showed that after large intraportal doses of dihydroergotamine in cats, it was sometimes possible to obtain depressor responses to small intraportal doses of noradrenaline, when similar doses of adrenaline were without effect. Nickerson and Goodman (1947) reported that the reversal of the adrenergic vasopressor effect by dibenamine was not altered by atropine, benadryl, and pyribenzamine, or by the anaesthetic used, and was due to vasodilatation

The mechanism by which the vasodepressor response to noradrenaline is produced is not clear Several possibilities exist (1) Constriction of the coronary arteries may occur Bacq and Fischer (1947) reported that extracts of human coronary nerves and arteries contain adrenaline, and they suggested that in certain diseases the synthesis of adrenaline is stopped at the stage of noradrenaline, which may have a vasoconstrictor action on the coronary vessels In direct contrast to this suggestion, Marsh, Pelletier, and Ross (1948) showed that noradrenaline increased cardiac output and coronary flow in isolated mammalian hearts (2) Constriction of branches of the portal vein in the liver may be produced. In two cats given dibenamine, the whole portal vein was closed, and when the blood pressure was stabilized large doses of noradrenaline still exhibited vasodepressor responses (3) Construction of the pulmonary arteries may take place A rise in pressure in the pulmonary vessels has been shown to induce a fall in the systemic circulation in a cat (Parin, 1947), this fall was the result of a cardiac component and a circulatory component The changes in the general circulation were due to vasodilatation, the spleen increasing in volume as long as its nerve supply remained intact was considered probable that pressoreceptor reflexes arise in the pulmonary artery, and it is possible that the depressor response to noradrenaline may be produced by this mechanism. In all the experiments with pentobarbitone, reversal to a pressor response did not occur immediately but was maximal after about an hour, when the vasodilator action of adrenaline still existed It is not known whether excess of pentobarbitone affects the fall in systemic pressure induced by a rise in pressure in the pulmonary arteries

SUMMARY

- 1 Large doses of *l* and *dl*-noradrenaline produced vasodepressor responses in cats given ergotoxine or dibenamine No such action was noted in rabbits
- 2 The mechanism by which this fall of blood pressure is produced has been studied. It is probably not due to vasodilatation, as is the normal adrenaline reversal response
- 3 Intravenous injection of pentobarbitone converted the depressor response to a rise of blood pressure in cats under chloralose

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MIRACIL CLINICAL TRIAL ON PATIENTS INFECTED WITH SCHISTOSOMA HAEMATOBIUM AND S MANSONI

BY

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A previous paper (Hawking and Ross, 1948) has described the toxicology, absorption, and excretion of miracil D in monkeys and in healthy volunteers. The present paper records a clinical trial of this compound on patients infected with Schistosoma haematobium and/or S mansoni. A preliminary note on these findings was published by Blair, Hawking, and Ross (1947)

Miracil D is the hydrochloride of 1-methyl-4- β -diethylaminoethylaminothioxanthone

It was synthesized by Mauss, and shown by Kikuth, Gonnert, and Mauss (1946) and Kikuth and Gonnert (1948) to have considerable therapeutic activity for mice and monkeys infected with S mansoni. The compound is administered by mouth. Subcutaneous or intramuscular injection causes considerable local irritation, and when given intravenously the toxicity is much greater than when given orally

As shown in the previous paper, it is rapidly absorbed from the alimentary canal, and 2½ hours after a single dose of 0.2 g to an average man the concentration in the blood rises to about 1 mg per litre. Only about 7 per cent of the drug is excreted in the urine, and little appears in the faeces, presumably about 90 per cent is degraded in the body. There is little tendency for the drug to accumulate. Deliberate prolonged overdosage in animals produced degenerative changes in the

liver and the renal tubules in a few animals, but these were usually much less than would have been expected

Organization of therapeutic trial

The present investigation was carried out at Salisbury, Southern Rhodesia, beginning in the second half of 1947 Patients with active schistosome infections were selected for the trials. Urines and stools were collected and examined for the pre sence of eggs and miracidia. The urines were exammed by collecting the terminal specimen of urine, centrifuging it, and examining the resultant deposit for eggs Originally the faeces were examined by collecting material from the outer layers of the stool together with any blood or mucus present and making an emulsion, with pond water, in a 3-in × 1-in tube This emulsion was then filtered through a coffee strainer and transfered to a conical urine glass, further pond water was added, and the whole was allowed to settle The supernatant was poured off, fresh water was added, and this washing process was repeated several times. Finally the resultant deposit was examined microscopically for the presence of eggs Later, improved methods of diagnosis were used both for urine and faeces, depending on the hatching out of miracidia from the washed deposit and their identification with a hand-lens under indirect illumination (Gorman, Meeser, Ross, and Blair, 1947, Meeser Ross, and Blair, 1948) Most of the therapeutic trials were made on young patients who were passing eggs in large numbers, infections in adults are less satisfactory for this purpose, since excretion of eggs is often intermittent

Previous to treatment each patient was examined clinically and weighed stripped. The age in Africans can be ascertained only approximately. Before commencing treatment a haemoglobin estimation was

made using a Newcomer standard (acid haematin) measured with a Klett-Bio colorimeter, a white blood corpuscle count and a differential leucocyte count were also made. The drug was given as uncoated 100-mg tablets (kindly made up by Messrs Burroughs Wellcome and Co) followed by a drink of water. With patients receiving once-daily doses it was usually given at 2-3 pm, about two hours after the midday meal, but occasionally it was given at 10 am. Doses were not given on Sundays. All doses were given under the personal supervision of one of the writers.

After treatment the patients were followed up at 7-day intervals for at least sixteen weeks. In some of the groups these weekly examinations were interrupted for four weeks by absence during the school The successful follow-up of so many of the school-children was greatly assisted by the helpful co-operation of Messrs C M Drury and F G Loveridge, successive headmasters of the Salisbury African School At each follow-up a specimen of urine and/or stool was examined by the methods already described Patients were considered "negative" if there were no eggs, or only calcified eggs were present. Patients were classified according to whether (1) active miracidia could be hatched (A), (2) living eggs were found but no miracidia hatched (E), (3) only dead eggs were present (D), (4) no eggs could be detected (O) A haemoglobin estimation and a white cell count were also made If the white cell count was altered significantly from the previous count, then a differential cell count was done to obviate the insidious onset of agranulocytosis or any other type of cell change

-CLINICAL RESULTS

The patients treated may be divided into five series, some of which may be subdivided into groups

First series of patients

The patients of the first series were divided into five groups The first group consisted of three adult male Africans employed in the laboratory, their ages were 30-45 years. The second group was made up of ten male African schoolboys, their ages ranged from 14-17 years, average 16 years, and their weights ranged from 40 to 64 kg, average 53 kg One boy in this group, who felt unwell on the fourth day, stopped treatment after one dose of 50 mg and three doses of 100 mg The third group consisted of thirteen male African school-boys, their ages ranged from 14-17 years, average 15½ years, and their weights from 33 to 66 kg, average 54 kg. The fourth group consisted of seventeen Eurafrican (coloured) schoolchildren, thirteen males and four females, whose ages ranged from 10-17 years with an average of

13 years As there were considerable differences in weight in this group, a demarcating line of 40 kg was taken, those over 40 kg (six boys weighing 41 to 68 kg, average 49 kg) forming one subgroup, while those less than 40 kg (seven boys and four girls weighing 30 to 40 kg, average 35 kg) formed another The fifth group consisted of one male European, aged 17 years

Of the forty-four patients under treatment, in the first group two had S haematobium infections and one S mansoni, in the second group, five had S haematobium infections and five had double infections of S haematobium and S mansoni, in the third group, two had S mansoni infections, nine had S haematobium infections, and two had double infections, in the fourth group, fifteen had S haematobium infections and two had double infections, and in the fifth group there was a single S haematobium infection

The results of treating the first series of patients are shown in Table I

It must be remembered that in schistosomiasis egg production as discovered by examination of the excreta may frequently be intermittent. During a series of repeat examinations of the same patient negative findings are not uncommon This may be due to a number of factors, such as ageing, relatively infertile worms ceasing to produce eggs regularly, or advanced damage of bladder and bowel with consequent fibrosis which makes the passage of eggs more difficult Thus in Group III one case was positive, negative, and then positive, and a second case negative, positive, and again positive at the end of treatment, four weeks later and eight weeks later respectively Furthermore. although reinfection during the time of the followup was unlikely, it was by no means impossible, and after eight weeks cases classified as "relapses" may possibly have been reinfections. These factors make the assessment of cure at any one time From Table I it can be seen that 12 infections (1 of S mansoni and 11 of S haematobium) were apparently cured—ie, no miracidia or fresh eggs were found twelve weeks after treatment, but in 9 of these 12 infections the excretion of eggs had initially been light or irregular number of patients cured was smaller than the number of infections cured, since 9 patients initially had double infections, and one species might disappear while the other remained The results of treating these double infections were as follows, among the eight patients followed for twelve weeks, not one remained negative for both infections although three became negative for S haematobium alone and one for S mansoni alone

TABLE I SUMMARY OF THE EFFECTS OF TREATMENT BY MIRACIL D (1ST SERIES) H indicates S haematobium, and M indicates S mansoni infections

7		Average	ļ		-	No of patients passing no living ova/No treated	passing no	living ova/No	treated		
~ ~	patients	weight	Doses	End of treatment	eatment	After 4 weeks	weeks	After 8 weeks	weeks	After 12 weeks	weeks
	1	0		Н	M	Н	M	H	M	Н	M
1 🖽	3 (2H, 1M)	565	200 × 1 150 × 1 200 × 1 200 × 13	2/2	0/1	2/2	0/1	2/2	0/1	2/2	0/1
5	10 (10H, 5M)	53	50 × 1 100 × 134 300 × 12	1/10 (6/9) *	1/5 (1/4)	3/9 (3/8)	3/4 (3/3)	3/4 (3/3) 1/9 (1/8)	2/4 (1/3)	2/9 (3/8)	1/4 (1/3)
	13 11H, 4M)	54	50 × 1 200 × 18–22	1/11 (7/9)	1/4 (0/4)	2/11 (2/9)	1/4 (1/4) 3/11 (4/9)	3/11 (4/9)	0/4 (0/4)	4/11 (3/9)	0/4 (0/4)
1 = `	11 (11H, 2M)	35	200 200 200 200 200 200 200 200 200 200	1/17 (6/16)	0/2 (0/1)	3/17 (3/16)	0/2 (0/1)	0/2 (0/1) 3/17 (3/16)	0/2 (0/1)	3/17 (4/16)	0/2 (0/1)
	(H)	59	200 × 19 £ 50 × 1 200 × 18	1/1		1/1		0/1		0/1	
1 🚊	44 (41H, 12M)			6/41 (19/34)	2/12 (1/9)	11/40 (8/33)	4/11 (4/8)	4/11 (4/8) 9/40 (8/33)	2/11 (1/8)	2/11 (1/8) 11/40 (10/33) 1/11 (1/8)	1/11 (1/8)

(a) 1 boy infected with S harmaroblum received only 1 dose of 30 mg, and 3 doses of 100 mg(b) 1 patient with a double infection could not be followed up

*Brackets refer to cases from which hatched miracidia could be obtained

TABLE II

SUMMARY OF THE EFFECTS OF TREATMENT (2ND SERIES)

Boys given up to 11 daily doses of 0 6 g during 15 days we $E = L_1 ving \ eggs \ D = Dead \ eggs \ O = No \ eggs \ or \ miracidia \ M = S \ mansoni$ A = Miracidia hatched and active E = Living eggs D = Dead eggs

	16	O A A M	0404404004	4/13
	15	041	ООШФФОШОШШ	5/13
eatment	14	041	00D44D40m4	6/13
nd of tr	6	044	0004404044	6/13
after er	8	O 40 W	000404004	6/13
Excretion of ova at number of weeks after end of treatmen	7	ОЩК	0004404044	6/13
mber of	9	044	0004<0DDmm	6/13
a at nu	'n	о Ч Ш В В	M000404040	7/13
n of ov	4	AAA	0000400040	10/13
excretio	3	ОКЫ	ADOOP#000	7/13
	7	ppinO	ОДОДКОШЩКШ	6/13
	-	AM AM	DAMOAA444	
	Mıracıdıa	++ AM AM	++++++++ ++++++++ +++++++++	
Initial state	Ova	+++	+ +++++++++++++++++++++++++++++++++++++	ematobium)
	RBC	+++ +++ +++	+++++++++++++++++++++++++++++++++++++++	Proportion not passing living eggs (haematoblu
Total dosc	mg /kg	77 83 46	35 35 61 61 84 88 83 83 83 84	assing livi
To	E9	300	04004466-6 0400440080	not į
7 89) 29 C	13 17 17	28725254955	rtion
Z		1 2 3 Also	4 2 9 2 0 1 2 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	Propo

TABLE III

All males except Nos 16, 17, and 18 Children given 0 3, 0 4, 0 5, 0 6, 0, 0 6, 0 7, 0 7, 0 6, 0 6 g on successive days (per 45 to 55 kg) Symbols as in Table II SUMMARY OF THE EFFECTS OF TREATMENT (3RD SERIES)

	15	40000000000440	12/18
	14	Q400 040000m044m00	12/18
tment	13	00000044000000044400	13/18
Excretion of ova at number of weeks after end of treatment	12	O40000400000A4000	13/18
fter end	11	000000400000004400	14/18
weeks a	10	000000MP000001 000	15/17
ber of v	6	0000004 000000 000	15/17
at num	8	000000m40 0000 000	14/16
of ova	7	00000 40000 0 100	11/13
cretion	9	000000440 000 000	13/15
Ä	5	0	
	4	0	
	3	00	
	7	щO	-
	*.	404m0m4m44m440m 404	4/18
e 748	Mıracıdıa	+++++++++ ++++++++++++++++++++++++++++	(u
nitial stat rch 31 19	Ova	++++++++++++++++++++++++++++++++++++++	ematobium
In Mar	RBC	++++++++++++++++++++++++++++++++++++++	Proportion not passing living ova (hae
Total dose	mg /kg	24888888888888888888888888888888888888	passing li
Tot	20	650 650 650 650 650 650 650 650 650 650	not
Age	<u>'</u>	\$200 E 4 4 8 5 4 4 4 5 5 5 8 6 8 8 6 8 8 6 8 6 8 6 8 6 8 6 8	ortion
Ş	?	155 176 177 178 178 178 178 178 178 178 178 178	Prop

* 3 days after last dose

If the results are reckoned in terms of individual patients, and the type of infection is disregarded, then out of forty-three patients treated only eight patients no longer passed eggs twelve weeks after treatment had finished, two of these were in Group I, one in Group II, three in Group III, and two in Group IV On the other hand, if the cases are judged by the criterion whether or not miracidia could be hatched from the excreta (satisfactory data on this subject are not available for all the patients), it is found that at the end of treatment 19 out of 34 cases of haematobium infection had become negative Two weeks after treatment 15 out of 26 cases were negative, at four weeks after treatment the results by this test (8 negative out of 33) were similar to those judged by the presence or absence of living ova (11 negative out of 40) results with infections of S mansoni were approximately the same whether judged by the presence of viable eggs or by the hatching of miracidia

From this series it was concluded that the therapeutic effect of miracil in daily doses of 0 3 g was not great. However, in view of reports from workers at Cairo that patients could tolerate larger doses of miracil than 0 3 g daily, and in view of the failure of miracidia to hatch in many of the cases after miracil treatment, further trials were instituted using higher levels of dosage.

Second series of patients

These consisted of thirteen African school-boys, who were given a daily dose of 0.6 g per day during 15 consecutive days (omitting the 11th, 12th, and 13th days on account of the weekend) Many boys complained of abdominal pain and vomiting (some of which may have been psychological in origin), so that attendance for treatment was irregular and only one of the boys, No 4, completed the full course Two of the boys had been treated with miracil six months earlier as part of the first series, No 2 having received 0.35 g and No 5 having received 4.25 g without any apparent benefit The details concerning these patients together with the results of treatment are shown in Table II

In spite of the great irregularity of the dosage, the therapeutic effects of the compound are much more definite than those observed in the first trial. The effects are most marked at three weeks after the end of treatment, at which time 10 out of the 13 boys had ceased to pass living eggs. After this some of the boys began again to show signs of infection. For the purpose of this paper, these cases are interpreted as relapses, although the possibility that some were reinfections cannot be excluded. By the sixteenth week 4 out of the 13

were still free from living eggs, these 4 boys had presumably been cured—i e, sterilized by the treatment. The therapeutic effect was not in direct proportion to the dose. The boys who were cured had received total doses of 77, 150, 98, and 55 mg per kg. In other boys—eg, No 2 and No 8—total doses of 83 and 91 mg per kg had exerted little or no definite effect on the excretion of living eggs.

Third series of patients

In view of the encouraging early results of this second series of patients, a third series was commenced The patients were again school-children A dose schedule for subjects of medium weight (45-55 kg) was planned as follows 1st day 0 3 g, 2nd day 04 g, 3rd day 05 g, 4th and 6th days 06 g (5th day omitted), 7th and 8th days 07 g, 9th and 10th days 0 6 g, total 50 g Adjustments were made for patients lighter or heavier than this These children took the drug much weight-range better than those of the previous series did, even though the average daily dose was greater actually received total doses ranging from 40 to 104 mg./kg, average 90 mg/kg, the average daily dose was about 8-10 mg/kg The details about these patients and the results of treatment are given in Table III In addition six similar children (five infected with S haematobium and one with S mansoni) chosen for controls were given placebo tablets of sodium citrate dved vellow so as to resemble miracil, the details of these are given in Table IV for comparison after the end of treatment, 13 out of 15 treated patients who were examined no longer passed live eggs, only 2 of those examined still showed signs of an active infection Fifteen weeks after treatment 9 out of 18 appeared completely free from infection, and 3 others passed only occasional dead One patient had never responded to treatment, while the remaining five showed a temporary response but the infection reappeared One patient (No 28) who was also infected with S mansoni continued to pass ova in the faeces The six controls (Table IV) continued to pass large numbers of living eggs throughout the period of observation, apart from the fact that a girl (No 32) with a double infection of haematobium and mansoni ceased to pass mansoni ova in the faeces

Fourth series—maximum tolerated doses of miracil

In order to discover the maximum amounts of miracil which could be tolerated, larger doses were given to patients with *haematobium* schistosomiasis who had been put in hospital for some other

TABLE IV
SUMMARY OF THE EXCRETION OF OVA IN SIX UNTREATED CONTROL CHILDREN, CHOSEN AT RANDOM FROM THE
THIRD SERIES

Symbols	as	ın	Table	П
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Age Wt		Ir	utial sta	ate	Ex	cretio	n of	ova a	t num	iber o	f wee	ks aft	er end	i of t	reatm	ent	
No	and sex	kg	RBC	Ova	Mıra- cidia	1	2–5	6	7	8	9	10	11	12	13	14	15
32	13♀	37 5	++	+ +M	++	A OM		A	A OM	0	A OM	A	A OM	A	A OM	E	A
33 34	17a 14a	53 46	++	+ + +	+ + +	A E	_	A O	A A	A	A	A	A	A A	A A	A A	OM A A
35 36	148 158	46 37	+	 	+	Ā		Ă A	Ā	A A	A	A A	A A	Ā	A A	A A	A A
37	168	43	_	O +M	+	O AM	-	0	O OM	O AM	O OM	O AM	O AM	O AM	O AM	O AM	O AM
Propo	ortion n	ot passi	ng livin	g ova		0/6		1/5	1/6	1/6	1/6	0/5	0/6	0/6	0/6	0/6	0/6

reason As shown in Table V, three patients were given 04 g three times a day for about 9 days (one patient only for 6 days), and three were given 06 g three times daily for 6 days Only one of these patients, No 40, showed any toxic effect that might have been due to the drug, he complained of abdominal pain and vomited on the fifth day, the significance of this vomiting is uncertain Before treatment began, miracidia could be hatched out from the urine of five of these patients the last day of their treatments, the urines of two of these five patients were free from eggs, the urines of the other three contained apparently living eggs but the miracidia did not hatch Another patient, No 44, was given 06 g twice daily for two days, soon after the first dose on the second morning he vomited The dose was reduced to 04 g once daily for two days, nausea was felt On the fifth day he was given 03 g twice and he had nausea and vomited Four days later the urine still contained eggs but miracidia could not be hatched Four other patients (Nos 45-48, not in hospital) were given once-daily doses as shown, which were increased during the five days of treatment to a maximum of 16 g Two of these (46 One (No and 47) had no toxic symptoms at all 45) weighing 54 kg complained of abdominal pain on the 4th and 5th days after doses of 1.2 g and 16 g respectively, he vomited after the last dose, without bringing up much of the compound patient (No 48, weighing 32 kg) had abdominal pains, nausea, and vomiting on the 4th and 5th days after doses of 08 and 10 g respectively the day following treatment miracidia could be hatched from the urines of three out of the four patients Six of these patients have been followed for 15 weeks, at the end of this period, none passed living ova

Fifth series of patients

Four miscellaneous patients were treated according to various schedules. The details about these are shown in Table V. In the two European children (Nos. 51 and 52), the skin was stained yellow by the drug. Three of these patients seemed to have been cured, one relapsed (or was reinfected) after 11 weeks.

Toxicity

The patients were questioned daily when given their dose for any toxic effects of the treatment, but with Africans it is generally difficult to evaluate reports of minor symptoms which may be exaggerated or concealed. The symptoms of possible toxicity during this investigation were still more difficult to evaluate because they tended to be more pronounced during the less intense courses than in the more intense ones.

In the first series (treated with 0.2-0.3 g daily) two boys reported slight or moderate nausea, each on one occasion, but its relation to miracil is uncertain. One fairly old man in Group I suffered more severe symptoms. He began taking miracil on July 21. On Aug. 2 he vomited, and for the next two days there was nausea, lack of appetite, and weakness. On Aug. 4 the drug was stopped (after 12 doses totalling 2.1 g) but was started again soon after. On Aug. 5 he felt all right, haemoglobin 86 per cent, WBC 4,200 per cumm as at beginning of treatment. On Aug. 7 and 8 nausea, anorexia, and vomiting, but he continued at work these two days. On Aug. 9 he could not come to work, cough, shortness of breath, slight disorientation, pulse rate 96. On Aug. 10 he

TABLE V SUMMARY OF THE EFFECTS OF TREATMENT (4TH AND 5TH SERIES) All males except 51 and 52, who were European girls Symbols as in Table II

	Symptoms		None	None	Pain and vomi- 5th day [ting	None	None	None	Nausea and vomiting	Abdominal pain:		None	Abdominal pain and nausea 2 days Slight vomiting 2 days		,
		15	ŀ		O _M			Q	0	0		0	0	00	D
	SG	41	1		0 0 0 0 0 0			0	0	0		0	D	00	0
	st do	13	1	,	0			Q	1	I		1	1	00	
- [Excretion of ova at number of weeks after last dose	12	1		O _M			0	0	Ĺ		1		00	V
	cs aft	=	0		0			0		Ω		0	Д	01	4
	week	2			0			Ω	Ω	Q		0	0	00	0
	r of	6	1		0			0	0	Ω		0	0	001	0
	nmp	- 8	1		<u>8</u> ∘			0	0	Α		0	0	001	<u> </u>
	at n	7	0		O O O O O O O O O			Ω	0	Ο.	Ω	Д	Ω	001	0
	ova	9	⋖		o _M			0	0	0	Ω	0	0	001	1
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ξ.	ctio	4	4		0		<u>-</u>	Δ	0	Ω	Ω	0_	0	<u> </u>	
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or of and oz, wild were European Euro	nıtıal state	Ova	FOUR ++	++	+ _M +	1	+	+	++	++	+	+	+ + +	+++	++
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	Total dose	mg /kg	190	298	121	190	202	212	74	107	107	105	112	91 154 97	92
	Tota	50	10 4	10 4	8 9	10 8	10 8	10 8	42	5.8	46	5 8	36	60 36	1 8
		Age	30	13	18	35	26	20	25	20	14	18	10	18	4
	ł	 8	38	39	9	41	42	43	4	45	46	47	48	49 50 51	52

*Figures in brackets show days after last dose

began to take food Aug 11, all right, chest, nothing abnormal discovered Aug 15, haemoglobin 63 per cent, WBC 3,500 per cu.mm These symptoms may have been due to other causes, although there was no clear evidence of an intercurrent infection, and they cannot be proved to have been due to miracil

None of the patients treated showed significant changes in the haemoglobin percentage or white cell counts during or after treatment. None complained of tinnitus (Africans might not report it even if present). There were no gross changes in the urine, slight albuminuria or haematuria was masked by the schistosomiasis. The European of the first series, Group V, became distinctly yellow in the skin and conjunctivae while taking the drug, but he had no other symptoms

In the second series there was a good deal of difficulty in getting the children to take the full doses (0 6 g once daily), they complained mainly of loss of appetite and abdominal pain and there seems to have been much deliberate absenteeism The doses were given as 6 tablets each of 100 mg, and this large number of separate pills may have had a bad psychological effect. One boy, No 4, took 66 g On the last day of his course (Feb 17) he complained of pain in the abdomen and inability to see clearly On Feb 24 he developed an acute generalized pruritus, which was severe during the short time it lasted The next day he showed a close herpetiform rash on the outstanding margin of his R trapezius muscle, perhaps an affection of the accessory nerve. In this connexion it may be recalled that Alves and Blair (1946) observed a herpetiform rash over the trapezius muscle in one of 100 Africans treated by their intensive course of sodium antimonyl tartrate Ritchken and Cantor (1947) noted an association between the administration of a similar intensive course of antimony and the onset of herpes zoster in five of their patients These cases of herpes and herpetiform rash had been attributed to the antimony, but in view of a similar occurrence during treatment with miracil it is possible that the herpes is really caused by the disintegration products of the dying schistosomes

In the third series most of the children registered no complaints except loss of appetite, but one child (No 28) felt ill after the penultimate dose Another complained of vomiting and of feeling generally weak on the 8th day after beginning treatment, but he took his other doses without incident

In the fourth series (which received the highest doses) the symptoms of toxicity were restricted to abdominal pain, nausea, and vomiting in some of

the patients, and they have already been described In the fifth series no toxic symptoms were observed

Concentration of miracil in the blood of patients

Three adult male Africans, who were infected with S haematobium (2) or S mansoni (1) were given miracil D by mouth at 8 am each day for 6 days Blood was withdrawn at appropriate intervals and the concentration of miracil was determined according to the method of Latner, Coxon, and King (1947) as used in the work of the previous paper These patients, who were confined to bed, suffered no toxic effects They left hospital six days after treatment, at which time they were still passing viable eggs. The details of these estimations are shown in Table VI, which should be compared with Table II and Fig 2 of the paper by Hawking and Ross (1948) The blood concentrations reached in different individuals vary

TABLE VI

CONCENTRATIONS OF MIRACIL D IN BLOOD IN MG
PER LITRE

Daily]	lst da	у	2nd	day	4th	day	6th	dav
dose mg	2 1 hr	6 hr	24 hr	2 <u>1</u> hr	74 hr	21 hr	24 hr	21 hr	24 hr
100 200 300	0 33 0 89	0 62 0 77 1 20	0 42 0 68 0 96	0 53 0 82 1 20	0 24 0 32 0 90	0 32 0 37 1 10	0 32 0 52 0 78	0 52 0 60 1 40	0 28 0 44 1 20

somewhat, allowing for these variations the concentrations obtained in these African patients were similar to those found in the European volunteers. There was no definite tendency of the compound to accumulate in the blood

Patients treated with miracil A B, and C

Trials were also carried out on miracils A, B, and C, but as only a small quantity of each compound (less than 10 g) was available, which had been kindly supplied by Dr Mauss for another purpose, only one patient could be treated with each

Miracil A is the hydrochloride of 4-β-diethylaminoethylamino-1-methylxanthone

Miracil B is the hydrochloride of 8-chloro-4-β-diethyl-aminoethylamino-1-methylxanthone

Miracil C is the hydrochloride of 4-β-diethylaminoethylamino-1-methylxanthydrol

Miracils A and B are xanthones whereas miracil D is a thioxanthone Miracil C is the dihydro derivative of miracil A, miracil B differs from miracil A in containing a chloro substituent in the second benzene ring

These compounds were synthesized by Mauss, and their chemotherapeutic activity on expenmental schistosomiasis in mice and monkeys was investigated by Kikuth and Gonnert. They found that these compounds are well tolerated by mouth, when given subcutaneously they produce marked local irritation which occasionally leads to necrosis at the site of the injection, when given intravenously they are mactive except miracil D, which is not given by this route for pharmacological reasons When given orally for the treatment of mouse schistosomiasis miracil B is almost four times as active as miracil C and D and more than eight times as active as miracil A In the treatment of monkey schistosomiasis the relative activities are different Miracil D is the best preparation, miracil B produces an effect only in doses which cause vomiting, and miracil A, which is only slightly active in mice, shows clear-cut activity

TABLE VII
TOXICITY TESTS

The toxicity of the miracil compounds, when given orally to mice

Com	Maximum tolerated d	lose in mg per 20 g
Com- pound	Kıkuth and Gönnert (sıngle dose)	Sewell (four daily doses)
Miracil A Miracil B Miracil C Miracil D	6 7 20 10 13	5 10 5 5–10

in monkeys Miracil C occupies a mid-way position in activity in both mouse and monkey tests

The toxicity of these compounds was kindly determined for us at the National Institute for Medical Research, London, by Mr P Sewell, by giving oral doses of 5, 10, and 20 mg/20 g mouse daily for four successive days. His results are given in Table VII and compared with those of Kikuth and Gonnert, obtained by treating mice with a single oral dose. In view of the small number of mice used, the results obtained are in agreement

Clinical trial

The patients were young Africans Each patient is considered individually

A Weight 63 kg, infected with S haemato-bium, complained of blood in urine before commencing treatment, previously treated with antimony for schistosomiasis in January, 1947. He was treated with 6 daily doses of 100 mg miracil A by mouth in first week and 6 daily doses of 200 mg miracil A in second week. Total dose= 18 g

B Weight 66 kg, infected with S haemato-bium, this man had no symptoms at commencement of treatment. He was treated with 5 daily doses of 100 mg miracil B by mouth in first week and 5 daily doses of 200 mg miracil B in second week. Total dose= $1.5~\rm g$

C Weight 66 kg, infected with S haemato-bium, this man, who was very dull mentally, complained of vague "falling turns" for past three years. He was treated with 6 daily doses of 100 mg miracil C by mouth in first week and 6 daily doses of 200 mg miracil C in second—week. Total dose=18 g

These three patients had viable eggs in their urines when treatment began. They were examined at weekly intervals during treatment, and at 2 weeks, 4 weeks, and 8 weeks after the end of treatment. On each occasion viable eggs were found in the urine 'Neither these three patients nor the patients who were treated in determining the blood concentrations showed any toxic effects of the drugs

Concentrations of miracils A, B, and G in the blood of patients

Adult African patients who were in hospital suffering from some other condition as well as schistosomiasis were used in determining the blood concentrations of these drugs. The concentration of the drug was measured by means of a Spekker photo-electric absorptiometer according to the

TABLE VIII
BLOOD CONCENTRATIONS OF THE MIRACIL COMPOUNDS
AFTER A SINGLE ORAL DOSE OF 200 MG

Compound	Mg per lı	tre at various	times after
Compound	2] hr	6 hr	24 hr
Miracil A Miracil B Miracil C	0 16 0 10 Nıl	0 12 0 28 Nıl	Nıl Nıl Nıl

method of Latner, Coxon, and King (1947) for the estimation of miracil D It appeared that miracils A. B. and C could be estimated in the same way as miracil D For each drug the blood concentrations after a single dose of 200 mg were determined and these are shown in Table VIII blood concentrations of miracil A and miracil B were less than those observed after a single dose of miracil D (Hawking and Ross, 1948, Table II) if allowance is made for the marked individual variations between different patients No drug could be detected in the blood of the patient who had received miracil C, as there was no more miracil C available, this result could not be veri-The blood concentrations of miracils A and fied B were followed in patients who received daily doses of 100 and 200 mg for 5 days as shown in Table IX, the urinary concentration of the drugs was determined on the two days following cessation of treatment. The results are shown in Table The blood concentrations of miracil A and B are lower than those observed in patients treated

TABLE IX
BLOOD AND URINE CONCENTRATIONS OF MIRACILS
A AND B DURING FIVE DAILY DOSES BY MOUTH

Daily dose	Blo	od con		ons	Urine o	concen- s mg/l
of	1st	day	2nd	5th	6th	7th
compound	2] hr	6 hr	day 6 hr	day 6 hr	day	day
Mıracıl A 100 mg	0.20	0 10	0 40	0.32	20	Nil
Mıracıl A 200 mg	0 30	0 46	0 56	0 48	27	Nil
Miracil B 100 mg	0 16	0 04	0 08	0 28	1.2	Nil
Mıracil B 200 mg	0 24	0 10	0 30	0 34	2 1	Trace

with miracil D in similar dosage (Hawking and Ross, 1948)

Owing to the limited supplies of the compounds which were available the patients observed were too few to yield significant results, but they may provide preliminary indications for further investigations. In these doses no toxic effects and no therapeutic effects were observed. Judging by analogy with miracil D, however, it is probable that much larger doses would have been tolerated and these might have been therapeutically effective.

DISCUSSION

This investigation on the therapeutic value of miracil D is still in progress and it will probably be several years before a reliable evaluation of the compound can be made. The present paper reports the preliminary results which have been obtained for the information and guidance of workers who may be interested in this subject in other parts of the world.

The therapeutic effect of miracil D seems to depend much more on the intensity of the dosage than on its total amount. At 0.2 or 0.3 g per person (4 mg per kg) daily, the therapeutic effect is slight and dubious even though treatment be continued for over three weeks. At 0.5-0.7 g per person (over 10 mg per kg) daily the therapeutic effect is marked, and a high proportion of the patients can be sterilized.

These results may be compared with those reported from Egypt in three papers by Azim, Halawani, and Watson (1948), Watson, Azim, and Halawani (1948), and Halawani, Watson, Nor El-Din, Hafez, and Dawood (1948) They treated patients with doses up to 75 g during 8 days, but permanent cures were not usually produced except by the highest doses, toxic symptoms seem to have been more pronounced than they were in Rhodesia

The data about the possible toxicity of the compound are conflicting One boy (second series) suffered from generalized pruritus and a herpetiform rash after taking 66 g during 15 days, this may have been due to the drug or to disintegration products of dying schistosomes One man suffered from intense nausea, with vomiting, profound malaise, cough, and slight disorientation after taking about 14 daily doses totalling about 25 g, these symptoms may or may not have been due to the drug Some of the boys in the second series complained of nausea and colic, but many of these symptoms may have been psychological Of the 11 patients given the biggest doses, seven were all right and five suffered from nausea, vomiting, and abdominal pain, but in only one patient

were the symptoms severe enough to require reduction of the dose

Taking the patients as a whole, it may be concluded that nausea, vomiting, and abdominal pain may occur, but they are not serious and their frequency is not proportional to the dose certain that the other untoward effects are really due to the drug All these symptoms of toxicity seem to be idiosyncrasies depending on the patient more than on the size of the dose No uniform set of toxic symptoms has yet manifested itself The yellow staining which is seen with light skins is unimportant except from the cosmetic point of The insomnia, headache, giddiness, vertigo, excessive sweating, tremor, and twitching observed by Halawani and his colleagues (1948) have not been seen in our patients, nor has there been any evidence of disturbance of the heart Halawani, Newsome, and Wooton (1947) have shown that the blood concentration of miracil (and presumably also its toxicity and its therapeutic potency) is raised by impairment of kidney function, most of our patients were vigorous adolescents in whom the kidney function was presumably good

The ultimate value of a drug depends on the ratio between its therapeutic effectiveness and its toxic effects, but convenience of administration, expense, and the possibilities of alternative treatments have to be taken into consideration. With miracil D evidence has now been obtained that a large proportion of patients infected with S haematobium can be sterilized by doses which are tolerated by the great majority of persons. (The expense of manufacture may, however, be a handicap to widespread use)

The effect on S mansoni is not clearly indicated by the cases treated to date in S Rhodesia. In the first series of patients, 11 out of 40 cases of haematobium infections were cured, but only 1 out of 11 cases of mansoni infections. In the second series, patient 3 had a double infection, neither of which responded. In the third series, patient 28 had a double infection, haematobium ova disappeared temporarily from the excreta, but mansoni ova were found at all examinations (figures incomplete). There is a suggestion that S mansoni responds less readily than S haematobium.

Further judgment about the value of miracil must be withheld until greater experience has defined more accurately the maximum tolerated dose, has shown whether dangerous idiosyncrasies exist, and has enabled the therapeutic effect of large doses to be compared accurately with that of antimonials In any case, miracil is of great scientific importance as showing antischistosome action in a new series of organic compounds, and it has the practical advantage of being effective when given by mouth. A safe drug which could be given orally for the treatment of schistosomiasis would be of very great value in Africa It could be given in villages to out-patients, and administration could be left to non-skilled hands If the effectiveness and safety of miracil are confirmed by wider experience, village treatment of schistosomiasis free from the thraldom of an intravenous ritual will at last become a practical possibility

SUMMARY

- 1 Miracil D (1-methyl-4- β -diethylaminoethylaminothioxanthone hydrochloride) is a new synthetic remedy for schistosomiasis. It is given by mouth
- 2 Forty young persons infected with S haematobium were treated with total doses of approximately 80-100 mg per kg during periods of approximately three weeks, mostly as daily doses of 0.2 g per person (4 mg per kg) Twelve weeks later eleven of them were free from infection Eleven infections with S mansoni (mostly the same patients as those with S haematobium) were similarly treated, one of them became free from infection
- 3 Thirteen boys infected with S haematobium were treated with doses ranging from 35 to 150 mg. per kg during fifteen days, given (irregularly) as daily doses of 06 g per person. Three weeks after treatment ten out of thirteen no longer passed living ova. Four months later four of the boys appeared permanently cured.
- 4 Eighteen school-children infected with S haematobium were given total doses of about 90-100 mg per kg during ten days, mostly as daily doses of 0.5-0.7 g per person (about 10 mg. per kg) Six weeks later fourteen out of sixteen no longer passed living ova, fifteen weeks later twelve out of eighteen appeared to be permanently cured Fifteen other patients (many adults) were given amounts up to 1.6 g daily
- 5 In most patients the compound was well tolerated Symptoms of toxicity consisted mostly of abdominal pain, nausea, and vomiting They were not directly related to the size of the dose and seemed to depend at least partially on idiosyncrasy of the individual patient.
- 6 Miracil D appears to exert a valuable therapeutic effect on infections of S haematobium in doses which are well tolerated by most patients

The dose should be at least 10 mg per kg per The effect of maximum tolerated doses on infections of S mansoni is not yet known

7 Three single patients were treated with miracil A, B, and C respectively in total doses of 15-18 g during two weeks No toxic or therapeutic effects were observed at this dosage, which is probably much less than the tolerated one

8 In patients taking about 3 mg miracil D per kg daily, the blood concentration at 2½ hours after the dose was 0 3 to 0 8 mg per 100 ml In patients taking miracil A and miracil B, the blood concentrations were similar to (or lower than) those taking miracil D. In a patient given miracil C, the compound could not be detected in the blood

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Morris, medical director, Southern Rhodesia, for access to clinical material and laboratory facilities, to the principals of the schools involved for their kind cooperation, and to Drs Mauss, W Kikuth, and R Gönnert for information and material

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ANTIMALARIAL COMPOUNDS AS ANTAGONISTS OF ADENOSINE

BY

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The possible antagonism of the action of adenosine on some tissues by antimalarial drugs has been studied with the object of obtaining some information on the mode of action of this type of drug

METHODS

Antagonism of adenosine

The following techniques were used in these experiments —

- (1) The isolated hen's caecum —The action of adenosine on this tissue (Barsoum and Gaddum, 1935) was studied before and after the addition to the bath of known concentrations of antimalarials. Although it was possible to demonstrate an antagonism between these compounds and adenosine by this method, satisfactory results were not obtained because of the intense relaxation of the caecum produced by the antimalarial substances themselves
- (2) The measurement of the duration of the auriculoventricular (a v) block produced by intravenous administration of adenosine in the anaesthetized guinea-pig (Drury and Szent-Gyorgyi, 1929) The guinea-pig's heart is not so sensitive to adenosine as the hen's caecum but this method has the advantage that the antimalarials do not alter the electrocardiogram (e c g) in sub-toxic doses

Three series of experiments were carried out with this technique

(a) In the first series, hereafter called "acute," the compounds under test were administered by slow intravenous injection. Guinea-pigs of 250-400 g bodyweight were anaesthetized with pentobarbitone (nembutal), given intraperitoneally. A cannula was inserted into one of the jugular veins and the animals maintained under artificial respiration during the experiments by means of a Palmer's Starling Universal pump for small animals, 1 10-4 or 1 10-3 adenosine in normal saline was administered intravenously by the jugular vein cannula, never more than 1 c c of adenosine solution being injected. Electrocardiograms were taken, lead II, immediately after the administration of adenosine by

means of a Cossor electrocardiograph modified for fast heart rates

In each experiment a series of doses of adenosine from 0 1 to 0 3 mg/kg was first administered in order to produce varying degrees of a v block. The cannula was then washed with the solution of antimalarial and the drug slowly infused by means of a motor-driven syringe which delivered the solution at the rate of 0 02 ml/min. This infusion was maintained for 30 min. after which the cannula was washed with adenosine solution and a second series of adenosine injections was administered in doses large enough to produce measurable heart blocks.

The dose of antimalarial was calculated in mg /kg /min and adjusted by varying the concentration according to the body-weight of each animal At least 3 guinea-pigs were used for each dose level of antimalarial

This series was used mainly for the selection of chemical types able to antagonize the action of adenosine

(b) In the second series the compounds were administered orally in a dosage regime similar to that used by Davey (1946) for the assessment of antimalarial activity against P gallinaceum in chicks

Batches, usually of 6 guinea-pigs each, were used for each dose level of every compound tested. The animals received two doses daily for 3½ days, a total of 7 doses. The drugs were administered in solution in water, or in fine suspensions when insoluble, by means of a stomach tube. After the 7th dose, the guinea-pigs were anaesthetized with pentobarbitone and a series of adenosine injections was administered as described above.

These experiments will be referred to as "chronic" later on

(c) Some experiments were carried out with heart-lung preparations of guinea-pigs. The method was essentially that described by Cruickshank (1945) for the rat, 25 ml of heparinized guinea-pig's blood were used in the apparatus. The elastic peripheral resistance was adjusted to give a pressure of 80-100 mm. Hg in the aortic cannula, e.g. g records were taken with leads placed in the superior vena cava and in the apex of the heart. Adenosine was injected into the venous cannula of the preparation

Assessment of the adenosine action and of the antagonistic action of antimalarials

The action of adenosine in normal and treated guineapigs was assessed by the duration of the a v block as recorded in the electrocardiograms obtained during experiments. The duration of this action was measured as the time which elapsed between the first and last P wave not followed by a ventricular complex. No attempt was made to measure the duration of these effects by the inspection of the duration of the P-R intervals. From these results, the doses of adenosine which would have produced a v blocks of 5 seconds' duration were calculated by graphical methods.

An attempt to compare the intensity of the adenosine antagonism shown by the substances under test was made by calculating the doses of antagonists which would reduce the sensitivity of the guinea-pigs to adenosine to one-half of the normal (see Tables II and III)

The results so obtained were only semi-quantitative and can only be taken as a rough measurement of the intensity of the antagonism

Antimalarial activity

The data on the antimalarial activity of the drugs used in this work was obtained in our laboratories by Dr D G Davey using chicks infected with *Plasmodium gallinaceum* (Davey, 1943) Most of these results have already been published in the papers dealing with the synthesis of the compounds in question

Materials

The substances used in this work can be classified in the groups indicated in Table I In this Table the chemical constitution and the trivial name or code number of each compound is given. In the text these numbers and names will be used in preference to the full chemical names. In each group substances have been selected with as low and as high antimalarial action as possible.

Most of the substances used in our work were prepared in these laboratories (Curd and Rose, 1946a, Curd, Davies and Rose, 1946, Curd, Davies, Owen, Rose, and Tuey, 1946 Curd, Richardson, and Rose, 1946, Curd and Rose, 1946b, Basford, Curd, and Rose, 1946, Curd and Rose, 1946c, Curd and Landquist, 1948, Curd, Landquist, and Rose, 1947) Others were obtained from Professor A R Todd (Hull, Lovell, Openshaw, Payman, and Todd, 1946, Hull, Lovell, Openshaw, and Todd 1947) Quinidine and hydroquinidine were a gift of Messrs Amsterdamshe Chininefabriek, Amsterdam The adenosine used was purchased from Messrs British Drug Houses, Ltd

RESULTS

(1) Antagonism in the hen's caecum

This antagonism was studied by measuring the relaxation of this tissue produced by known concentrations of adenosine in normal Tyrode's solution and in Tyrode's solution containing known quantities of antimalarial

Mepacrine usually had practically no visible effect on the caecum in concentrations of 1 10⁻⁷ to 1 10⁻⁶, but it decreased the action of adenosine in this concentration range. Higher concentrations caused an intense relaxation of the caecum and at the same time reduced still further the action of

TABLE I

CHEMICAL COMPOSITION OF SOME OF THE ANTI MALARIAL AND RELATED COMPOUNDS USED

Quinolines

Name or code number		Substituents
Pamaquin	6 8	OCH ₃ NH CH(CH ₃)(CH ₂) ₃ N(C ₂ H ₂) ₂
Chloroquine	- 4 7	NH CH(CH ₃)(CH ₂) ₃ N(C ₂ H ₅) ₂ Cl
5735	2 and 3 4 7	CH ₃ NH CH(CH ₃)(CH ₂) ₃ N(C ₂ H ₄) ₄ Cl
3738	2 4 7	OH NH(CH ₂) ₂ N(C ₂ H ₄) ₂ Cl

Biguanides Cl NH NH R_1 R_2

Name or code number	Paludrine	4430	5093
R ₁	CH(CH ₃) ₂	CH(CH ₃) ₂	CH ₃
R ₂	Н	CH3	Н

Arylguanidinopyrimidines

$$\begin{array}{c} NH \\ \parallel \\ NH - C - NH \\ N = \\ NH(CH_2)_nN(C_2H_2)_2 \end{array}$$

Code number	3349	3749	3742
R	Cl	Cl	CH ₃ O
n	2	3	2

Anilinopyrimidines

$$2 \left\langle \sum_{N=6}^{3} 4 \right\rangle$$

Code		Subs	utuents	
number	2	4	5	6
2666 3300 4070 3756 4316	(p)Cl C ₄ H ₄ NH (p)Cl C ₄ H ₄ NH CH ₃ (p)Cl C ₆ H ₄ NH (C ₄ H ₉) ₂ N(CH ₂) ₃ NH	CH ₃ CH ₃ (p)Cl C ₆ H ₄ NH —CH = CH — (CH ₃	CH = CH —	NH(CH ₂) ₂ N(C ₂ H ₆) ₂ NH CH(CH ₃)(CH ₂) ₃ N(C ₂ H ₆) ₂ NH(CH ₃) ₂ N(C ₂ H ₆) ₂ NH(CH ₂) ₂ N(C ₂ H ₆) ₂ (p)Cl C ₆ H ₄ NH

Pyrimidines

$$2 \stackrel{3}{\swarrow}_{N} \stackrel{4}{\longrightarrow}_{NH(CH_2)_nN(C_2H_6)_5}$$

Code number		Su	bstituents	
number —	2	4	5	n
4747 4419 3920 3448 4746 4420 4450 4184	CH ₃ CH ₃ NH ₂ NH ₂ NH ₂ CH ₃ CH ₃	CH ₃ NH ₄ CH ₃ CH ₃ CH ₄ NH ₂ CH ₃ CH ₄ CH ₄	CH ₃ CH ₂ C ₆ H ₅ CH ₁ CH ₂ —	2 2 2 2 2 3 3 3 3

adenosine Fig 1 summarizes the results of one of these experiments

Similar results were obtained with quinine, 3349, and 2666, but the intense relaxation of the caecum produced by each of these antimalarials made the assessment of the antagonism somewhat difficult For this reason this method was abandoned after a few trials with different antimalarial drugs

(2) Antagonism between adenosine and antimalarials on guinea-pig's heart

The duration of the a v block produced by the intravenous administration of adenosine is proportional to the dose. The minimum effective dose of adenosine, in guinea-pigs anaesthetized with pentobarbitone, was 0 1 mg/kg, and this generally produced a block of short duration during which one or two independent P waves were recorded. The average duration of this block-in a series of 40 guinea-pigs was found to be 0.67 sec. Blocks of 10-15 sec. duration were generally obtained with 0.3 mg/kg of adenosine. The mean dura-

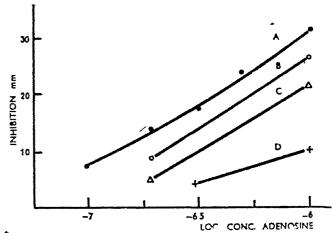


Fig 1—Influence of mepacrine HCl on the response of the hen's caecum to adenosine Abscissae—log concentration of adenosine Ordinates—relaxation of caecum in millimetres A—normal Ringer B—in mepacrine 10-7 C—in mepacrine 10 f D—in mepacrine 10 5 b

tions of the blocks produced in this control series with doses of adenosine from 0.1 to 0.3 mg/kg are given in Table III

TABLE II

MINIMAL DOSES WHICH ANTAGONIZE THE ACTION OF
ADENOSINE IN ACUTE EXPERIMENTS

Drug	Minimum antagonistic dose mg/kg/min	Dose which halves sensitivity to adenosine mg /kg /min
Quinine HCl Mepacrine HCl Pamaquin HCl Chloroquine H ₃ PO ₄ Paludrine lactate 3349 2666 Procaine Suramin Methylene blue Trypan blue Trypan red Sulphadimethyl- pyrimidine	0 75 0 25 too toxic at 0 25 0.25 1 25 3 0(?) 0 6 Inactive at 2 0 1 at 5 0 1 at 2 0 1 at 2 0 1 at 2 0 1 at 8 5	0 8 0 35 0 45 1 25 3 0(?) 0 9

A "Acute" experiments

Table II gives the minimum antagonistic doses of the compounds tested in this series and the doses which reduce the action of adenosine to one-half

Mepacrine

The duration of the blocks produced by adenosine after the continuous administration of

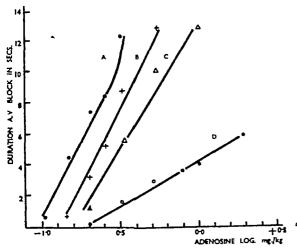


Fig 2—Acute antagonism between adenosine and mepacrine in the guinea-pig Duration of the a v block produced by adenosine in guinea-pig after a continuous injection of mepacrine HCl for 30 min Abscissae—log dose of adenosine in mg/kg Ordinates-duration of block in seconds A—before administration of mepacrine B—after 0 25 mg/kg/min mepacrine D—after 0 5 mg/kg/min mepacrine

01 mg/kg/min mepacrine for 30 min did not differ significantly from that obtained before the antimalarial had been given. An appreciable reduction of the duration of the action of adenosine was observed after the administration of 0.25 mg/kg/min mepacrine. The decrease in the effects of adenosine was more marked after 0.35 and 0.5 mg/kg/min of antimalarial had been given. Fig. 2 shows these results

This antagonism, however, is reversible. When the response to adenosine was examined at different times after the end of the infusion of mepacrine, it was found that the duration of the block became progressively longer, and in 90 min the adenosine effects were about the same as those of the control series (Fig. 3)

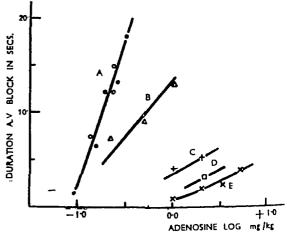


FIG 3—Duration of the a.v block produced by adeno sine before and at different times after the end of the continuous injection of mepacrine HCl of 0.5 mg/kg/min for 30 min Abscissae-dose of adenosine as log mg/kg Ordinates-duration of block in seconds A (dots)—before mepacrine E—immediately after mepacrine D—15 min after mepacrine C—30 min after mepacrine B—60 min after mepacrine A (circles)—90 min after mepacrine

Pamaquin

It was impossible to study the action of pamaquin by this method because of its high toxicity Doses as small as 0.25 mg/kg/min of pamaquin HCl were too toxic and caused bradycardia and right predominance in the ecg Larger doses of pamaquin HCl killed the guinea-pigs before the end of the infusion period

Chloroquine ~

Chloroquine is less toxic than pamaquin Doses from 0.25 to 1 mg/kg./min were administered without causing undue alterations of the e c g The

minimum doses of chloroquine that antagonize the action of adenosine are of the order of 0.25 mg/kg/min

Quinine

Quinine was mactive when administered in doses of 0.5 mg/kg/min. A good reduction in the duration of the a v block produced by adenosine was observed after doses of 0.75 mg/kg/min had been infused.

Paludrine

Paludrine was found to be active in doses from 10 mg/kg/min and the adenosine antagonism was very marked with 2 mg/kg/min. In some of these experiments the paludrine concentration of the blood was as high as 7 to 9 mg/l

3349

3349 was found to be even less active than paludrine in antagonizing the effects of adenosine in this "acute" series. Doses of 2 mg/kg/min were not antagonistic, doses of 3 mg/kg/min showed a clear antagonism but were too toxic to the heart. The e c g tracing showed cardiac irregularities and bradycardia, which made it impossible to assess the intensity of the antagonism.

2666

The antagonism to adenosine with this compound was more intense than with paludrine or 3349 but less than with quinine, mepacrine, or chloroquine Doses of 1 mg/kg/min of 2666 were strongly antagonistic, whereas 0.5 mg/kg had no action on the adenosine effects

Substances without adenosine antagonism

A selection of substances, some of them without antimalarial activity, were tested by this method Procaine, suramin, methylene blue, trypan red, trypan blue, and sulphadimethylpyrimidine were tested, none of these antagonized the adenosine effects

B "Chronic" experiments

The adenosine antagonism shown by antimalarials of different types was studied in more detail in this series of experiments. Details of these experiments are given in Table III, which also gives the doses of antagonists which halve the sensitivity of the animals to adenosine

Acridine derivatives mepacrine

The heart blocks produced by adenosine in guinea-pigs that had received seven doses of

mepacrine in 3½ days were shorter than those obtained in normal guinea-pigs

The minimum dose of mepacrine which showed this effect was 2×5 mg/kg daily Larger reductions in the duration of the adenosine effects were observed when the dose of mepacrine was increased from 5 to 40 mg./kg twice daily With the last-mentioned dose, the minimum effective dose of adenosine was 0.3 mg/kg, which produced the same effect as 0.1 mg/kg in normal guinea-pigs Details of the results of these experiments are shown in Table III and Fig 4

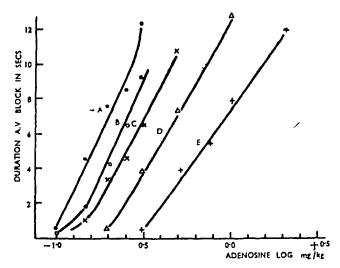


Fig 4—"Chronic" antagonism between adenosine and mepacrine HCl in guinea-pigs Duration of the a v block produced by adenosine in guinea-pigs treated by mouth with different doses of mepacrine Abscissae-dose adenosine as log mg /kg Ordinates-duration of block in seconds A—controls B—mepacrine HCl, 2 × 5 mg /kg /day C—mepacrine, 2 × 10 mg /kg /day D—mepacrine, 2 × 20 mg /kg /day E—mepacrine, 2 × 50 mg /kg /day

Outnoline derivatives

Pamaquin given by mouth as the methylene bis- β -hydroxynaphthoic acid salt was tolerated by guinea-pigs in doses up to 20 mg/kg twice a day for $3\frac{1}{2}$ days. A reduction of the duration of the adenosine block was obtained in guinea-pigs after treatment for $3\frac{1}{2}$ days with 2×10 mg/kg pamaquin salt, equivalent to 625 mg/kg base

Chloroquine diphosphate was antagonistic to adenosine in doses of from 2×5 mg/kg/day. The low toxicity of this substance allowed the investigation of its antagonism in a range of doses from 5 to 50 mg/kg twice daily. Doses of adenosine that produced blocks of 5 sec duration in guineapigs treated with pamaquin or chloroquine did not show any significant difference between the intensities of the antagonism produced by these two substances

ANTAGONISM OF ADENOSINE BY ANTIMALARIALS ADMINISTERED ORALLY TWICE DAILY FOR THREE AND A HALF DAYS (7 DOSES) TABLE III

Compound	Dose (mg /kg)	Number of anımals	0 10 M	0 10 0 15 Mean du	Adenosii 0 2 ration ol	the injected 0.25 (f. auriculo (seconds)	Adenosine injected mg /kg 0 0 15 0 2 0 25 0 30 0 50 1 00 Mean duration of auriculo-ventricular block (seconds)	g 0 50 ular blo	1 00 ck	Dose which halves sensitivity to adenosine mg /kg	Mınımal dose for full antımalarıal actıvıty mg /kg
Controls (no drug)		8	190	4 58	8 12	9 65	12 58	-	l		
Quinine (dihydrochloride)	50 150 200	0000	4000	12 04 00 00	2 48 1 6 2 1 0 5	36 32 10	53 49 44 138	10 15 7 0 2 86	16 06 11 7 9 4	09	9
Quinidine (base)	50 100	5	000	39	53	75 2 57	109		11	09	40
Hydroquinidine (base)	100	9	0.5	2.1	3.5	09	7.5		1	100	80
Mepacrine dihydrochloride	2004	9899	0 31	3 01 0 45 0 12	4 36 3 21 1 75 —	6 55	9 23 6 54 3 72 0 65	15.5 7.15 3.46	12.7	14	40
Pamaguin methylene bis β-hydroxy- naphthoate	20	821	0 9	3 4 1 86	53	77	9 5 6 88	ſI		6	4
Chloroquine diphosphate	20 20 20 20 20	9999	00 00	21 23 00	3.75 3.55 0.6 0.0	59 505 26 00	8 5 7 4 4 0 0 0	11.4 7.8 4.6	13.2 7.0	6	5
5735 dıhydrochloride	205	9	0 43	12	17	34	37	64	11	33 ,	08
3738 base	20	9	0.0	2 8	52	8 05	10 8	1	1	70	Inactive at 400
Paludrine lactate	10	99	00	2.5	68 54	83	117	11	11	15	5
4430 acetate	200	9	000	12	3 9	7 25 5 9	12 8 7 5	11	11	40	, 20
5093 hydrochloride	20 50	99	2 38 1 8	42	734 67	9 32 9 8	12 25 15 9	11	11	No action	Inactive at 160

TABLE III (continued)

ANTAGONISM OF ADENOSINE BY ANTIMALARIALS ADMINISTERED ORALLY TWICE DAILY FOR THREE AND A HALF DAYS (7 DOSES)

Compound	Dose (mg /kg)	Number of anımals	0 10 M	0 15 fean du	Adenosi 0 2 ratjon o	ne inject 0 25 f auricul (second	Adenosine injected mg /kg 10 0 15 0 2 0 25 0 30 0 50 1 00 Mean duration of auriculo-ventricular block (seconds)	ig 0 50 cular bic	1 00 ock	Dose which halves sensitivity to adenosine mg /kg	Minimal dose for full antimalarial activity mg /kg, *\$\frac{3}{4}\$
3349 base	20 50 75 100	2049	000	2 0 0 16	394	5 16 2 04 0 0	6 56 3 58 0 28 0 0	6.5 1.5 0.0	1 8 0 0 0 0	20	40
3672 dihydrochlonde	100	9	0.8	2.9	46	8.9	10 2 1 0	4.2	13.2	30	200
3742 base	20 20	9	0 12	5 5 2 48	89	10 8	14.4	11	11	Inactive	Inactive at 200
2666 dıhydrochloride	200 200 200	999	1.5	4 5 1 9 0 0	66 11 00	97 3 65 3 0	13.2 7.62 4.5	13.9 6	111	06	80
3300 dihydrochloride	50 75 100	5 6 6	0.35	4 16	5 66 0 8 0 66	7 28 2 55	74 60 17	6.56	6.74	75	200
4070 base	20 100 100	999	0 86	54	82 22 11	105	14 5 7 0 4 3	13 4 8 1	 17.72	09	Inactive at 120
3756 base	20 20	99	0.5	6 26 2 5	93	104	152 92		11	No action	Slightly active at 40
4316 dihydrochloride	100	9	00	2.1	36	09	8.2	1	1	100	40
4747 base	50	9	1	4 1	72	10.5	11 05		1		Trace activity at 240
4419 base	20	9		1 8	2.7	4 4	62		1		08
3448 base	20	9	1	1	0.4	1	2.8	61	107		100
4746 base	20	9	90	3.9	45	72	9.5	 			Trace activity at 400
4420 base '	20	9	00	0.4	12	26	5.5	1	1		80
4450 base	20	4	ı		1	0 27	0 35	49	40		80
4184 base	20	4			03	1 02	3.1	69	10.9		120
						1				,	

The antagonism to adenosine shown by some derivatives of chloroquine such as 5735 and 3738 was weaker than that of the parent compound. The order of intensity of the antagonism shown by these substances was practically parallel to that of their antimalarial activity. Chloroquine and pamaquin, both of which have high antimalarial activity, antagonize adenosine better than 5735, which has low antimalarial activity. 3738, which has hardly any antimalarial action, did not modify the action of adenosine.

Cinchona alkaloids

The cinchona alkaloids studied in this series were quinine, quinidine, and hydroquinidine In the experiments with quinine it was observed that the antagonism to adenosine was marked in guineapigs for 2 hours after the last dose of alkaloid, but in experiments carried out later than that adenosine produced blocks of about the same duration as in the controls. As it is known that quinine is metabolized in the body fairly quickly, it was thought that these differences in the results were due to the rapid disappearance of the drug from the blood For this reason the dosage schedule for cinchona alkaloids was modified in the following way The six guinea-pigs used for each dose level received their seventh dose of drug at the usual time, about 930 a.m., and three of them were used before 12 noon The remaining three received an eighth dose at about 1 30 pm and were used immediately after With this modification of the dosage regime the results for both groups were about the same

Quinine and quinidine were adenosine antagonists in guinea-pigs when doses larger than 50 mg / kg were administered twice daily. The intensities of the antagonism to adenosine produced by these two alkaloids were practically the same, and it is interesting to note that both are equally active against experimental malaria.

Hydroquinidine, which as an antimalarial is about half as active as quinine, produced a milder action than the latter

N¹-phenyl-N⁵-alkylbiguanides

In the series of drugs of the paludrine type we selected one drug without antimalarial activity (5093), and compared it to 4430 and paludrine itself (4888) The inactive compound was found not to antagonize the action of adenosine even in the maximum tolerated doses

Paludrine antagonized adenosine when doses from 2×10 mg/kg/day were administered to guinea-pigs. Unfortunately it was not possible to

increase the dose beyond 2 x 20 mg/kg/day, all guinea-pigs treated with this dose survived this treatment for 4-5 days, but although a small increase of the dose to 2 x 30 mg/kg/day did not kill any animal during the first 11-2 days it produced a 100 per cent mortality if the treatment was maintained for a longer time. This high toxicity of paludrine, when given repeatedly to guinea-pigs, contrasts with its moderately low toxicity when given in single doses Similar results have been found by Butler, Davey, and Spinks (1947) in other laboratory animals, and it is probable that they are due to the formation and accumulation of some highly toxic metabolite of paludrine during the period of repeated administration

The minimum dose of 4430 which antagonized the action of adenosine was 2×30 mg/kg/day. This drug has a lower antimalarial activity than paludrine and at the same time it is a weaker antagonist.

Arylguanidinopyrimidines (Curd and Rose, 1946a)

A similar gradation of antagonistic activity was found among the substances of this type, and their antagonistic activity ran parallel to their antimalarial action 3349 has an antimalarial activity and a capacity to antagonize adenosine similar to those of paludrine 3672 is a weaker antagonist and a weaker antimalarial, and finally 3742, which has no action on malarial parasites, does not antagonize adenosine

Anilnopyrimidines (Curd and Rose, 1946b, Curd, Davies, and Rose, 1946, Curd, Davies, Owen, Rose, and Tuey, 1946, Curd, Richardson, and Rose, 1946)

The prototype of this group of substances (2666) was the first substance synthesized by Curd and Rose that showed antimalarial activity in chicks The results of its clinical trials were disappointing, and according to Adams it is not active in human malaria (quoted by Curd, Davey, and Rose, 1945)

This group of substances was the only one in which it was impossible to find any relation between antimalarial activity and adenosine antagonism. Two members of the group, 3300 and 4070, are devoid of antimalarial activity but they were, nevertheless, good antagonists of adenosine. 2666 itself is a substance with a fairly good antimalarial activity, but it inhibited the action of adenosine more intensely than 4316, which is a better antimalarial. On the other hand 3756, which is inactive against experimental malaria, did not show any appreciable antagonism to adenosine.

Alkylpyrimidines (Hull, Lovell, Openshaw, Payman, and Todd, 1946, Hull, Lovell, Openshaw, and Todd, 1947)

Because only small samples of these compounds were available, they were not tested in a full range of doses For this reason it was neither possible to study the relative order of the intensities of their antagonisms nor to compare these with their antimalarial activities All of them were tested at 2×50 mg/kg/day, which as a rule with most antimalarials produced a measurable antagonism It is interesting to note that compounds 4747 and 4746, which are inactive against P gallinaceum, did not antagonize the action of adenosine under the conditions of the test Under the same conditions. all the other compounds tested in this series produced a clear antagonism. The details of the results of these experiments are reproduced in Table III

Relation between antimalarial activity on chicks and adenosine antagonism

As already stated above, within all groups of substances, except the 2666 type, there is a parallelism between their antimalarial activity and their power to antagonize adenosine, as expressed by the dose which halves the sensitivity of the animals to this substance. In comparing the effects of the substances selected as prototypes for each group it was found that mepacrine was one of the best antagonists of adenosine, in spite of the fact that it is one of the weakest antimalarials in chicks. Among the other substances, however, the best antimalarials are among the best antagonists, and substances with low antimalarial activity are among

TABLE IV

Adenosine :	antagonism	Antımalarıal
"Acute"	"Chronic"	activity
Mepacrine Chloroquine Quinine 2666 Paludrine 3349	Pamaquin Chloroquine Mepacrine Paludrine 3349 Quinine 2666	Pamaquin Chloroquine Paludrine 3349 Mepacrine Quinine 2666

those which antagonize adenosine feebly Table IV gives the orders of antimalarial activity and intensity of the antagonism shown by these substances in the "acute" and "chronic" experiments

C Antagonism in the heart-lung preparation

In the experiments reported above it was found that paludrine antagonized adenosine in both "acute" and "chronic" experiments. When the intensity of its "chronic" antagonism was compared with that of other prototype substances it was found that the order of intensity of their antagonism was about the same as that of their antimalarial activity. However, in the "acute" series this parallelism was not maintained and under these conditions paludrine was found among the worst antagonists (Table IV)

Another fact noted was the delayed toxic effect of paludrine when administered repeatedly. A likely explanation of this toxic action may be the production and accumulation of some toxic metabolite during the prolonged administration of paludrine

It is also possible that the difference in the results obtained with paludrine in the "chronic" and "acute" experiments may be due, not to the drug antagonizing adenosine by itself, but to its being converted during the dosing period into a metabolite which does antagonize adenosine. Even when the paludrine content of the blood was as high as 7–9 mg/l in the "acute" experiments, the antagonism was not intense, but during the brief period of slow administration of the drug only a small proportion of the injected material could have been metabolized into the hypothetical active substance

The possibility of such a metabolite being formed was investigated in the heart-lung preparation of the guinea-pig in the following way e c g records of the action of several small doses of adenosine (from 25 μ g) were taken before and after the addition of paludrine to the blood of the preparation The blood was then replaced by the same volume of fresh normal blood, which was left to circulate for 15 min, this "washing out" of the drug was repeated and finally the blood was replaced by blood from guinea-pigs which had been treated orally with 2×20 mg/kg of paludrine for $3\frac{1}{2}$ days This last change of blood was repeated and 25 ml, of blood from paludrine-treated guinea-pigs were again put in the venous reservoir A further series of adenosine doses was injected 15 min after the last change of blood

With normal blood in the preparation the injection of about 25 μ g adenosine into the venous cannula produced blocks of about 5 sec duration. The addition of paludrine to the blood of the preparation caused no reduction in the duration of the effects of adenosine, but a significant reduction of these effects was observed when the blood

of paludrine-treated guinea-pigs circulated in the preparation

The concentration of paludrine in the blood of the treated guinea-pigs and that in the blood to which paludrine had been added was estimated by the method of Spinks and Tottey (1946) In some experiments, such as that reproduced in Table V, the concentration in the blood of the treated animals was smaller than that in the blood to which paludrine had been added, but nevertheless the blocks produced by the same doses of adenosine were shorter when the blood of paludrine-treated animals circulated in the preparation

TABLE V

ANTAGONISM TO ADENOSINE BY ANTIMALARIALS IN
HEART-LUNG PREPARATIONS OF GUINEA-PIGS

Expt No	Blood (25 ml) and drug	Duration of a v block in secs with doses of adenosine, in µg			
		25	35	50	100
2	Normal blood	1 4	_	94	
	Ditto and mepacrine HCl 0 02 mg Normal blood (after 3	00	_	68	
	changes)	3 4		11 9	
	Ditto and mepacrine HCl 0 2 mg	00	_	50	11.3
13	Normal blood	3 4	_	12 8	-
	Ditto and quinine HCl 0 05 mg	3 6		12 6	~~
	Ditto and quinine HCl	00	_	_	00
	Normal blood (after 3 changes)			8 4	14 0
4	Normal blood		6.2	11 3	
	Ditto and paludrine (1 027 mg/l) Blood of paludrine-		68	10 2	
	treated guinea-pigs (conc 0 82 mg/l) (after 2 changes with blood of paludrine-treated guinea-pigs)		29	56	_
	1	[~	į.	l	Į

These results were compared with those obtained when mepacrine or quinine was added to the blood of the preparation

The duration of the heart block produced by adenosine was reduced when mepacrine was added to the blood in concentrations of $80 \mu g / 100 \text{ ml}$ This antagonism was observable so long as blood containing mepacrine circulated in the preparation When the drug was "washed out" by two or three changes of blood the duration of the action of

adenosine returned to normal values - Similar results were obtained with quinine in concentrations of the order of 2 mg /100 ml Table V gives the results of some of these experiments

DISCUSSION

The antimalarial action of a number of chemical substances has been compared with their power to antagonize the responses of some mammalian tissues to adenosine. In most of the series of compounds tested a correlation has been observed between the inhibition of responses to adenosine and the antimalarial activity. This may mean that antimalarial action in some way involves interference of the drug with a process, essential to the parasite, in which adenosine or a derivative thereof is concerned.

No idea as to the nature of this hypothetical process can be gathered from the experiments reported here. It is unlikely that the processes in mono-cellular plasmodia in which adenosine or its derivatives are concerned can be closely related to the processes which we have been studying in mammalian tissues.

This study of the antagonism of the action of adenosine in some tissues by antimalarial drugs was undertaken because adenosine forms part of coenzymes, such as, for instance, flavine adenine dinucleotide. It was already known (Madinaveitia, 1946) that the antibacterial action of some antimalarial compounds on Lactobacillus casei was antagonized by riboflavine. It has been suggested that this effect is due to the similarity of the spatial configuration of these drugs to that of the vitamin (Curd, Davey, and Rose, 1945). This structural resemblance has also been claimed to be connected with the parasiticidal activity of these drugs (Curd and Rose, 1946a).

On the other hand the alternative hypothesis that an interference with an adenosine-containing enzyme system might result in antimalarial activity has led to the preparation of successful antimalarial compounds (Hull et al., 1946, 1947) biochemical relationship between adenosine derivatives and antimalarials may be found in the observation (Hellerman, Bovarnick, and Potter, 1946) that adenylic acid and adenosine triphosphate prevent the inhibition which mepacrine causes in the recovery of oxygen uptake by washed Plasmodium lophurae in the presence of glucose The fact that some enzymes contain both adenosine and riboflavine makes both points of view on the mode of action of antimalarial substances compatible

It is unlikely that L casei uses riboflavine as such in its metabolic processes It is more probable that the micro-organism builds up some coenzyme from the free riboflavine present in the medium, possibly by combining it through a polyphosphate group with adenosine It is conceivable that the antagonistic effect of riboflavine in L casei assays is not due to the vitamin itself, but to an adenosine-containing coenzyme into which the free riboflavine is incorporated prosthetic group of d-amino-acid oxidase is such a flavine adenine dinucleotide (Corran, Green, and Straub, 1939) and is known to antagonize the inhibitory action of mepacrine on the enzymatic deamination of d-amino-acids (Wright and Sabine, 1944)

On the other hand it has been shown (Haas, 1944) that mepacrine also inhibits cytochrome reductase by an irreversible reaction with the protein, here the drug has been shown to compete with the prosthetic group of the enzyme, because its action is prevented by the addition of alloxazine mononucleotide. In this coenzyme riboflavine is not combined with adenosine

All the antimalarial drugs which we have tested have been found to antagonize the particular actions of adenosine which we have chosen to study. From the circumstantial evidence already available and that now presented it appears that some relationship exists between the ability of chemical compounds to interfere with biological processes in which adenosine is concerned and their antimalarial action.

In spite of the fact that the results of the experiments reported above were only semi-quantitative, it was found (a) that antimalarial drugs have another action in common—i.e, they antagonize adenosine, and (b) that the intensity of this antagonism is proportional to their antimalarial action as measured in chicks infected with P gallinaceum

The relation of the two activities is best seen when the results obtained with a series of homologues are compared. The only exceptions to this rule were the anilinopyrimidines of the 2666 type among which no relation between antimalarial activity and adenosine antagonism could be found. It is noteworthy that although 2666 was found to be active in the chick test, the drug was tried clinically with negative results by Dr. A. R. D. Adams, of the School of Tropical Medicine of Liverpool (cited by Curd, Davey, and Rose, 1945). However, as the chick test is only an indication of possible antimalarial activity in man, and as there is an unpredictable variability in the susceptibility

of various species of plasmodia to the same drug (Davey, 1946), it is more than probable that the agreements and disagreements shown in Tables III and IV would have been different if another measurement of activity had been chosen

In comparing the plasmodicidal activity of the prototype of each group of substances with the intensity of their adenosine antagonism in the "chronic" series, it was found that mepacrine was the best antagonist, although it is not one of the more powerful antimalarials in the chick test. There is, however, a fairly close parallelism between the antagonism and the activity of the other substances

This relationship is not so close in the "acute' experiments. The most important exception was paludrine in the "chronic" series it was found among the more intense and in the "acute" series among the weaker antagonists. The difference in the results of the two series could be explained by the assumption that in the "chronic" experiments a metabolite with antagonistic action was formed from paludrine during the period of its administration.

At the end of the 30 min period of continuous infusion in the "acute" experiments, the paludrine concentration in the blood was sometimes as high as 9 mg/l. In spite of the fact that such high drug concentrations could be obtained only a slight antagonism could be demonstrated. It seems likely that during the infusion period only a small proportion of the drug was metabolized into the hypothetical substance responsible for the antagonism

It has also been suggested that paludrine itself does not possess plasmodicidal action when examined by a tissue culture method (Hawking, 1947, Hawking and Perry, 1948), but the serum of animals which have been treated with paludrine has a high plasmodicidal activity, from these results Hawking has deduced that the antimalarial activity of paludrine is not due to the drug itself but to some metabolite

The formation of metabolites with biological actions in the guinea-pig different from those of paludrine has been demonstrated in the heartlung experiments In these preparations the addition of small amounts of mepacrine and quinine to the blood reduced the duration of the block produced when adenosine was injected into the venous cannula If similar amounts of paludrine were added to the blood there was no reduction of the effects of adenosine, but substantial decrease of these effects was observed when blood from paludrine-treated guinea-pigs, possibly containing metabolites of the drug, circulated in the preparation (Table V)

This result cannot be explained by differences in the paludrine content of the blood from the treated guinea-pigs and that of the blood to which paludrine had been added. In some experiments these concentrations as measured by the method of Spinks and Tottey were the same for all practical purposes (This method consists in the acid hydrolysis of the base, after its extraction with benzene, to p-chloroaniline and subsequent diazotization and coupling of the latter It does not give any indication of the possible in vivo changes undergone in the side chain of paludrine, provided that they do not alter the solubilities and rate of hydrolysis to any great extent How alterations in the benzene ring would affect these results is difficult to predict)

It is interesting to note that in the acute experiments the antagonism shown by mepacrine was not permanent, and about 90 min after the infusion of the drug the action of adenosine returned to normal In heart-lung preparations the antagonistic action of mepacrine and guinine could be even more easily reversed by changing the blood containing drug for fresh normal blood. It appears that the antagonism to adenosine is due mainly to the drug which circulates in the blood and not to that which is fixed in the tissues

Pamaquin and paludrine, or more probably some metabolite of it, behave with respect to adenosine in the same way as the rest of the antimalarial drugs assayed This is in contrast to the failure of riboflavine to antagonize their action on L cases, a property common to the other antimalarials assayed Perhaps paludrine does not show such antagonism because under the in vitro conditions of the test its transformation into the substances responsible for its adenosine antagonism and antimalarial activity does not take place. One of the reasons which led Curd and Rose (1946d) to suggest that the antimalarial activity of paludrine was different from that of other types of antimalarials was this failure of riboflavine to antagonize it. In the light of the experimental evidence that paludrine can be converted in the blood of treated animals into some metabolite which does share with other antimalarials the property of antagonizing the action of adenosine, the case for such assumed difference in the mode of antimalarial action is somewhat weakened

SUMMARY

Antimalarial compounds antagonize the action of adenosine on the guinea-pig heart and on the hen caecum

A parallelism between the antimalarial activity and the power to antagonize adenosine has been demonstrated in most series of compounds examined

In heart-lung preparations it has been found that unlike the other antimalarials tested paludrine does not antagonize the action of adenosine However, the blood of paludrine-treated guinea-pigs did antagonize this action. This is taken as evidence that paludrine is metabolized to another compound with different pharmacological properties

The considerations which led to the testing of antimalarials as antagonists of adenosine are discussed

It is a pleasure to record grateful acknowledgment of the technical assistance rendered by Miss H Todd and Mr T Johnston

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THE EFFECT OF HEPATECTOMY ON THE ACTION OF CERTAIN ANAESTHETICS IN RATS

BY

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To investigate the role of the liver in the detoxication of anaesthetics, two main methods are available First, the liver may be damaged by some drug such as carbon tetrachloride, if the anaesthetic has a greater effect than before, it is probable that it is normally detoxicated by the This method is open to at least two objections In the first place, it is impossible accurately to assess the amount of liver damaged by this means, and further it is possible that the carbon tetrachloride has some action of its own on the central nervous system The second method, which we have used, is to remove by operation a large proportion of the liver and observe whether the effect of the anaesthetic is increased method has the advantage that the amount of liver tissue removed can be estimated fairly accur-

ately, and it is unlikely that the operation has any complicating sideeffects Higgins and Anderson (1931)described a method for partial hepatectomy in rats, and the high survival rate and quick recovery of the animals make it suitable for this type of investigation

METHOD

Albino rats weighing between 150 and 300 g were used Each rat was given a preliminary dose of the drug to be tested in the following manner Food was withheld overnight, and the following morning the drug was given to the animal by stomach-tube in a dose calculated according to body-weight At fre-

quent intervals the rat was laid on its side, and when it remained in this position it was considered to be asleep As soon as it got to its feet it was considered awake, and the sleeping-time was thus determined. At least five days later partial hepatectomy was carried out by the method of Higgins and Anderson (1931) The animals withstood the operation well, the mortality being about 10 per cent, and by the third day after operation appeared perfectly normal hours after operation they were fed on bread and milk and 20 per cent glucose Thereafter they were given the normal laboratory diet. On a chosen day after hepatectomy, the rat was given the same dose of drug as before, and the sleeping-time again determined in the same way. The experiment was planned to give at least 10 results on each of the following days after hepatectomy 6th-7th, 8th-9th, 10th, 12th, 15th, 17th-18th, 20th, 22nd, and 26th-28th sleeping-time of the rats on each of these days after

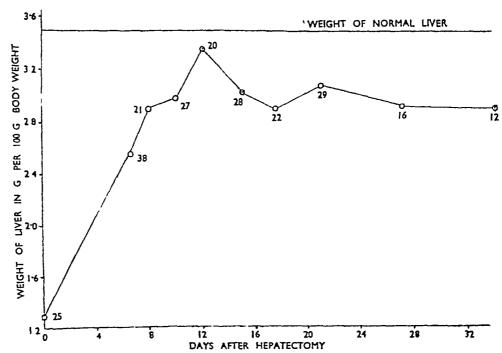


FIG 1—Graph showing regeneration of liver by weight after hepatectomy Numbers at each point show the number of rats used to determine each mean

operation could then be compared with their mean sleeping-time before operation

Immediately each experiment was finished the rat was killed and its liver removed, washed in saline, dried with filter-paper, and weighed No rat was used twice after hepatectomy

The drugs and doses used were chloral hydrate, 350 mg per kg, bromethol, 300 mg per kg, soluble phenobarbitone, 110 mg per kg, thiopentone, 50 mg per kg

RESULTS

1 Rate of regeneration of liver

In order to obtain an average figure for the amount of liver removed at operation a prelimin-

ary experiment was carried out on 40 rats. The animals were hepatectomized and the portion of liver removed was weighed. The animals were then killed and the remainder of the liver was dissected out and weighed. From these two weights, the mean percentage of liver removed was calculated, and found to be 62.2 per cent by weight (S D = 4.88 per cent). This figure is lower than that of 70 per cent found by Higgins and Anderson (1931). Expressed as g. per 100 g. body-weight of rat, the mean weight of liver left behind after operation in these 40 rats was 1.29 (S D = 0.28) and the mean weight of the whole liver was 3.49 (S D = 0.81).

TABLE I

DIFFERENCES IN MEAN SLEEPING-TIME OF GROUPS OF RATS ON VARIOUS DAYS AFTER HEPATECTOMY DEATHS
SHOWN IN COLUMN (IV) ARE INCLUDED IN THE FIGURES IN COLUMN (V) EACH AS 500 MIN SLEEPING-TIME

(1)	(ii)	(пі)	(iv)	(v)	(v1)
Number of rats	Day after hepatectomy	Mean sleeping- time in min before hepatectomy	Number of deaths due to effect of drug after hepatectomy	Mean sleeping- time in min after hepatectomy	Difference (v)-(111)
HLORAL HYDR	ATE (350 mg per kg))	<u> </u>		
16 10 10 10 10 10 10	6-7 8-9 10 12 15 17-18 22 28	171 1 162 0 85 2 85 7 209 4 78 0 176 3 147 0	4 6 4 3 7 1 0	346 1 373 7 277 4 341 0 384 0 112 9 74 8 97 5	+175 0 +211 7 +192 2 +255 3 +174 6 +34 9 -101 5 -49 5
ROMETHOL (300) mg per kg)		·····		
16 11 10 10 10 10 10	6-7 8 10 12 15 17-18 22 26	59 7 91 0 77 5 24 1 111 7 98 0 76 7 172 8	8 8 6 7 4 - 3 0	416 6 413 0 396 6 375 5 281 6 265 0 93 5 55 4	+356 9 +322 0 +319 1 +351 4 +169 9 +167 0 +16 8 -117 4
SOLUBLE PHENOI	BARBITONE (110 mg I	per kg)			
17 10 11 10 8	7–8 10 12 16 28	6 8 0 19 0 0	0 0 0 0	98 5 78 1 75 0 20 5 0	+91 7 +78 1 +56 0 +20 5
Thiopentone (5	0 mg per kg)		<u></u>		
3 10 8 10 8	4 8 12 15 20	133 3 94 1 12 0 86 8 92 0	2 3 2 1 1	573 3 314 0 227 0 212 4 110 0	+440 0 +219 9 +215 0 +125 6 +18 0

Fig 1 shows the mean weights of the liver (in g per 100 g bodyweight) of groups of rats on various days after hepatectomy will be seen that reproceeded 5+300 generation very rapidly during the first week after operation and that the liver had doubled its weight by the 7th day In our experiments the liver had not regained its original weight by the 34th day, whereas in Higgins and Anderson's experiments had ıt regained its weight by the 14th day

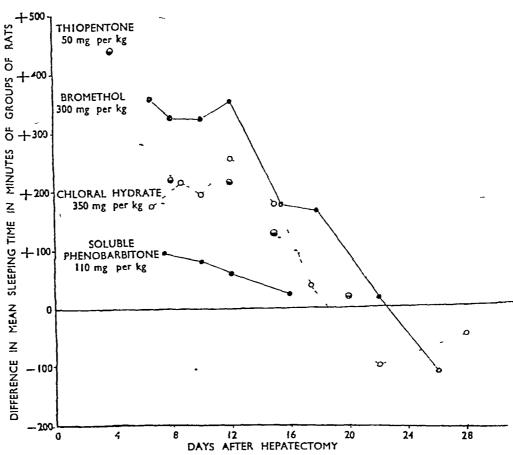
Brues, Drury, and Brues (1936) found that there was a fairly constant relationship between the weights of liver removed and that left behind, and therefore expressed the degree of liver regenera-

tion as a percentage of the original liver weight We have found that this method gives figures with a large scatter and that more consistent results are obtained by using the actual weight of liver in the rat in g per 100 g body-weight of rat

2 Effect of anaesthetics after hepatectomy

Table I shows the mean sleeping-times of groups of rats before and on various days after operation Column (vi) gives the difference between these two figures and shows whether the effect of the drug was increased or not. In the early days after operation the greatly increased effect of the drug resulted in the death of some animals. In order to include these results in the calculations it was necessary to consider them as if they had slept for a long period. Since rats rarely slept after any of the drugs for more than 400 min, death was considered as equivalent to the arbitrary figure of 500 min sleeping-time.

It can be seen from Table I that up to the 12th day after operation the effect of chloral hydrate was greatly increased. On the 18th day the effect was only slightly greater than that before opera-



fore expressed the defore expressed the dedays after hepatectomy

tion, and on the 22nd day it was actually less Similarly, Table I shows that by the 22nd day the effect of bromethol was almost the same as before, and was less than before operation on the 26th The results for soluble phenobarbitone show that there was a definite though smaller increase in sleeping-time after operation, and that this increase had almost disappeared by the 16th day It will be seen that, with the dose of phenobarbitone used, animals rarely slept before operation It was not possible to use a larger dose, which would produce sleep in most animals before operation, without causing a large number of deaths Table I shows that by the 20th day the effect of thiopentone was almost the same as before hepatectomy These results are expressed graphically in Fig 2

In order to make sure that the increased effect of these drugs after hepatectomy was due to the loss of liver substance and not to the operative interference or anaesthetic, control experiments were done for all four substances. A group of rats was given the usual dose of the drug, five days later laparotomy was carried out, and on the fourth day after operation a second dose of the

TABLE II

TABLE SHOWING THAT THERE IS NO CORRELATION BETWEEN DEGREE OF LIVER REGENERATION BY WEIGHT AND DIFFERENCE IN SLEEPING-TIME AFTER OPERATION FIGURES ARE PERCENTAGES OF ANIMALS IN EACH GROUP

	(i)	(n)	(111)	(vt)	(v)		
Drug	Difference in sleep- ing-time	ın teri	ms of wei	anımals g ght of live body-weig	er in g		
	in min	<2 5g	2 5-3 0g	3 0-3 5g	>3 5g		
	No increase +1-200 +201-400 +>400	30 25 40 5	50 18 16 16	36 36 18 10	37 18 27 18		
Brom- ethol 300 mg/kg	No increase +1-200 +201-400 +>400	17 28 38 17	47 17 23 13	22 34 22 22	16 12 36 36		

drug was given as before It was found that the increase in the mean sleeping-time was negligible

We observed that rats before hepatectomy never slept for longer than 200 min with any of the drugs. If the drug had a greater effect the animal died. After hepatectomy, however, the drug often caused rats to sleep for much longer periods. For instance, of 144 normal rats receiving bromethol, the highest sleeping-time was 190 min. Of 69 hepatectomized rats receiving bromethol, 10 slept for longer than 350 min without dying. This observation is consistent with the idea that after hepatectomy the detoxication processes are slowed.

3 Correlation of effect of drug with degree of liver regeneration

We were interested to see if there were any correlation between the degree of regeneration of the liver and its ability to detoxicate anaesthetics there were a correlation, we should have expected animals with marked liver regeneration to be less affected by the anaesthetic than those whose livers had only regained a small proportion of their former weight In Table II animals are arranged in four groups according to the weights of their livers at death (columns (ii), (iii), (iv), and (v)) Each of these groups is divided into four classes according to the difference in sleeping-time after hepatectomy The figures in each column are the percentages of rats in each group. If there were a correlation, we should expect that a high percentage of animals with small liver-weights would have a much increased sleeping-time after operation, and conversely a high percentage of those

with large liver-weights would have little or no increase in sleeping-time. It is clear from Table II that there is no such trend For instance, for chloral hydrate, 45 per cent of rats whose liver weighed less than 25 g per 100 g showed an increased sleeping-time after operation of more than 200 min, whereas the same percentage (45) of animals whose livers weighed more than 35 g also had an increase of more than 200 min sleeping-time Similarly, for bromethol, 55 per cent of animals with less than 25 g liver per 100 g and 72 per cent of animals with more than 35 g per 100 g showed an increase of sleepingtime after operation of more than 200 min Hence the degree of regeneration of liver by_weight is no guide to its ability to detoxicate these drugs

DISCUSSION

Chloral hydrate and bromethol are usually said to be detoxicated in the liver. Thus, Mukerji and Ghose (1940) found that, in dogs with livers dam aged by carbon-tetrachloride, there was a well marked excretion of free chloral in the urine after oral administration of the drug, whereas in normal dogs all the urmary chloral was conjugated There is, however, little definite evidence that bromethol is detoxicated in the liver, though it has been claimed that it can cause transient liver damage (Bourne and Raginsky, 1931, Coleman, 1938) Our experiments show that both these drugs are detoxicated in the liver, though the possibility that partial hepatectomy affects the renal excretion of them has yet to be excluded Table II shows that there is no correlation between the degree of liver regeneration by weight and its ability to detoxicate It is clear that the restoration of the enzyme systems which break down or conjugate these drugs is independent of the restoration of the mass of liver tissue

Most observers state that phenobarbitone is not detoxicated in the liver Cameron and De Saram (1939) found that there was no significant pro longation of its action in rats after acute liver damage caused by carbon tetrachloride Masson and Beland (1945) stated that the anaesthetic effect of phenobarbitone was not increased by partial hepatectomy, whereas after bilateral nephrectomy rats slept for over twice as long as the controls Similarly, Hirschfelder and Haury (1933) found that bilateral nephrectomy markedly increased the effect of phenobarbitone in rabbits These and other workers, therefore, consider that soluble phenobarbitone is eliminated through the kidney and not detoxicated to a significant extent by the liver It is, however, not possible to recover more

than 25 per cent of administered phenobarbitone in human urine (Halberkann and Reiche, 1927), so that 75 per cent is still to be accounted for Our experiments show that partial hepatectomy-increases the effect of soluble phenobarbitone up to the 16th day after operation. The possibility that hepatectomy affects the renal excretion of the drug in some way has yet to be excluded, but a probable explanation is that the drug is detoxicated to some extent in the liver

It will be seen from Table I that the increase in mean sleeping-time with phenobarbitone on the seventh day after operation (90 min) is much less than that with chloral and bromethol. This is possibly due to the relatively smaller dose of phenobarbitone used

The evidence about thiopentone is conflicting Richards and Appel (1941) found that its effect in rats was not increased after liver damage by carbon tetrachloride, and Scheifley (1946) found that its effect was not increased by partial hepatectomy. On the other hand, Schideman, Kelly, and Adams (1947) found that partial hepatectomy, liver damage with carbon tetrachloride, and the production of an Eck fistula all markedly increased the effect of thiopentone. They also showed that the drug was broken down when incubated in vitro with liver slices. Our results support these authors' conclusions that thiopentone is detoxicated in the liver.

Fig 2 shows that between the 22nd and 26th day the liver appears to be better able to detoxicate chloral hydrate and bromethol than before hepatectomy. Further work is necessary to confirm and elucidate this phenomenon. It is impossible to say whether the same holds good for phenobarbitone, since, with the dose used, animals seldom sleep before operation.

SUMMARY

- 1 A method is described of determining whether anaesthetics are detoxicated in the liver, by using partial hepatectomy in rats
- 2 The anaesthetic effect of chloral hydrate, bromethol, soluble phenobarbitone, and thiopentone is increased by partial hepatectomy. This increased effect is no longer present for chloral hydrate by the 17th, for bromethol by the 22nd, for soluble phenobarbitone by the 16th, and for thiopentone by the 20th, day after operation
- 3 There is no apparent correlation between the regeneration of the liver by weight and its power to detoxicate these drugs

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THE PHARMACOLOGY OF THE OPTICAL ISOMERS OF AMIDONE (2-DIMETHYLAMINO-4 4-DIPHENYLHEPTAN-5-ONE)

RY

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-A brief description of the preparation of the optical isomers of amidone has already been published (Thorp et al, 1947b), in which it was shown that it is possible to prepare these substances by resolution of 1 1-diphenyl-3-dimethylammovaleronitrile, the penultimate compound in the synthesis of amidone, and conversion of the optically active nitriles into the corresponding ketones. Some brief pharmacological results were given in that note, but it is now possible to describe the pharmacology of these substances more fully

Analgesic activity

The relative analgesic activities of d-, l-, and dl-amidone were determined in comparison with that of morphine. The method used has already been described by the author (1946), it was a modification of the radiant heat stimulation method of Hardy, Wolff, and Goodell (1940). The comparison was performed on a group of forty rats on each of four days, and two dose levels for each drug were used. The animals were randomly arranged into eight groups of five rats, and the treatments changed on each day so that all rats

had received one dose of each drug at the completion of the test. The initial pain threshold was determined before injection, the drugs, dissolved in amounts of saline such that the appropriate doses were contained in 0.5 ml per 100 g of rat, were injected subcutaneously at intervals of 2 min. This interval was introduced in order to allow for the time taken in estimating the pain threshold subsequent to injection, so that responses might be measured at equal intervals

Previous tests had indicated that the effects of morphine and amidone upon the pain threshold of rats are maximal approximately 30 min after injection and that the curves for the duration of action of the two substances are almost identical A single determination of the pain threshold half an hour after injection was therefore used for estimations of analgesic potency

Table I shows the results obtained in the comparison of the analgesic activities of d-, l-, and dl-amidone with that of morphine

The equiactive doses in the sixth column were obtained by estimating the dose of each drug producing a 50 per cent increase in pain threshold,

TABLE $\, {f I} \,$ THE COMPARATIVE ANALGESIC ACTIVITIES OF THE OPTICAL ISOMERS OF AMIDONE BY THE RAT METHOD

Drug tested	Dose No		Mean 100	crease in pain	Equiactive doses	Activity ratio	
	mg /kg	of rats	Per cent	Standard error	Slope	mg/kg (±%SE)	(morphine = 1)
d-Amidone hydrochloride	10 20	20 20	34 9 34 8	3 45 3 65	nil		
I-Amidone hydrochloride	0 8	20 20	31 9 61 8	3 19 5 75	169 8	1 02 ±10 45%	2 1
dl-Amidone hydrochloride	1 2 2 4	20 20	41 0 63 5	3 78 8 80	74 8	1 58 ±12 05%	1 36
Morphine sulphate	2 0 3 0	20 20	46 8 63 7	4 72 10 01	96 0	2 16 ±11 15%	1 0

the slope value estimated for each regression line being used

The activity ratios are the ratios of the reciprocals of the equiactive doses taking the value for morphine as unity. The three values for the slopes were tested for similarity, and it can be shown that, for P=0.95, the differences between them are not statistically significant. The standard errors of the equiactive doses in the fifth column do not preclude the possibility that l-amidone is twice as active as the racemic isomer

The results show that in the rat *dl*-amidone hydrochloride is probably a rather more powerful analgesic drug than morphine sulphate, and that the activity of the *laevo* isomer is very much greater than that of the racemic form

The dextro isomer failed to produce a graded increase in pain threshold when given in large doses, and the observed rise in pain threshold was probably not due to true analgesic action. In doses comparable with those of dl-amidone, d-amidone is without effect upon the pain threshold in rats. It is therefore very probable that the laevo isomer is twice as active as the racemic form and the dextro compound inactive.

Toxicity

The toxicity of the optical isomers of amidone was determined by intravenous injection into mice, the method of calculating the LD50 value described by Karber (1931) being used. The doses were always given in 0.25 ml of physiological saline and the results obtained are given in Table II

. It will be seen that the LD50 values for the three isomers are of the same order of magnitude The actual values obtained do not, however, agree with

those previously reported by the author (1947a, 1947b), which were between 10 and 18 mg/kg. We have repeated acute toxicity experiments on mice of two different strains and upon different days, and found that the absolute values of the LD50 for amidone isomers are subject to wide variations, although the results are comparable within individual experiments

Finnegan et al (1948) found the LD50 of dlamidone hydrochloride to be 92 ± 0.4 mg/kg in rats upon intravenous injection, but pointed out that the toxicity in acute experiments is very much greater than that of morphine, whereas the values obtained with smaller doses in a subacute experiment were much more similar

These findings show that the acute toxicity of amidone is not due to the central nervous depressant property since this is almost absent in the dextro isomer. The acute toxic effect is the result of sudden cardiovascular failure, and the LD50 value is determined largely by the resistance of the animals to a critical fall of blood pressure of brief duration.

There is evidence, however, from the results given above that the toxicity of *l*- and *dl*-amidone is enhanced by the depressant action these drugs exhibit since the LD50 value for *d*-amidone is just significantly greater than that of the other isomers

When cats and dogs were anaesthetized with pentobarbitone sodium and arranged for recording blood pressure and respiration, the following results were obtained

d-Amidone—In both dogs and cats similar effects were observed after intravenous injections of d-amidone Small doses of 10 or 20 mg/kg caused a brief fall in blood pressure of 40-80

TABLE II

THE ACUTE TOXICITIES OF THE OPTICAL ISOMERS OF AMIDONE UPON INTRAVENOUS INJECTION INTO MICE

d-Amidone HCl		l-Amic	ione HCl	dl-Amidone HCl		
Dose mg/kg	Mortality	Dose mg/kg	Mortality	Dose mg /kg	Mortality	
22 64 32 00 45 28	0/20 14/20 20/20	11 32 16 00 22 64 32 00 45 28 64 00	0/20 2/20 9/20 14/20 17/20 20/20	8 00 11 32 16 00 22 64 32 00 45 28	0/20 2/20 3/20 8/20 14/20 20/20	
LD50 = 29 9 mg /kg Fiducial limits (P = 0 95) 27 47 to 32 58		Fiducial lin	26 02 mg./kg nits (P = 0 95) to 29 55	Fiducial lin	24 24 mg /kg nits (P = 0 95) to 27 76	

mm Hg There was no depression of the respiration and when a series of doses of 2 mg/kg were given at 15-min intervals a similar fall in blood pressure was produced with each injection. The duration of each depressor response was from 1 to 2 min. Death resulted in all animals after the injection of 7-10 mg/kg of d-amidone and was characterized by a precipitate fall of blood pressure to approximately one-third of the normal level. This effect was associated with temporary respiratory arrest followed by Cheyne - Stokes respiration, and death usually occurred in 20 min or less. These effects were not modified by section of both vagi in dogs.

When guinea-pigs in which the brain and upper part of the spinal cord had been destroyed were supplied with artificial respiration and arranged for recording blood pressure, injections of 1 mg /kg of d-amidone caused a marked fall in blood pressure similar to that observed in anaesthetized dogs or guinea-pigs

These experiments show that the principal cause of the toxic action of d-amidone is a precipitate fall in blood pressure which is due to direct action upon the cardiovascular system

1-Amidone—The injection of l-amidone into the anaesthetized dog or cat caused effects which were markedly different from those described above. In the dog a first injection of 1 mg/kg of l-amidone caused a fall of blood pressure of approximately 50 mm. Hg lasting about 1½ min. The blood pressure then rose to a value slightly above the normal. The respiration showed marked depression and became irregular. A second dose of 1 mg/kg caused a much smaller fall in blood pressure followed almost at once by a prolonged increase of approximately 30 mm. Hg above the normal level.

The respiration showed a further degree of depression A third similar dose 10 min later caused no detectable fall in blood pressure, but a further rise in the general level. After both vagus nerves were cut and the animal left for an interval of an hour, injections of 1 and 2 mg/kg of l-amidone caused only a fall in blood pressure.

In the cat the blood pressure showed the same brief fall upon injection of the drug, followed by a return to a level slightly below the normal value Subsequent doses caused a progressively less marked initial fall, but a general lowering of the level of blood pressure followed

This initial fall in blood pressure has been examined by Shideman and Johnson (1948), who have shown that it becomes less upon subsequent injections and is a phenomenon exhibited by most analgesic drugs. They have termed the effect "acute vascular tolerance"

The effects of *l*-amidone were in general indistinguishable from those of *dl*-amidone described by Scott and Chen (1946), and, in our experiments upon animals in which the vagi were intact, cardiac slowing was also observed after injections of *l*-amidone

The acute effects of the amidone isomers appear to be due to two principal actions an immediate fall in blood pressure caused by direct action upon the heart and possibly also the vascular system, and, with the *l*- or *dl*-isomers, an additional depression of respiration and some slight degree of parasympathetic stimulation

Effect upon respiration

The effect upon respiration was compared in the rabbit by means of the respiration recorder described by Gaddum (1941) The recorder was

TABLE III

THE EFFECT OF THE OPTICAL ISOMERS OF AMIDONE UPON THE RESPIRATORY MINUTE VOLUME IN RABBITS

d-	d-Amidone HCl		-Amidone HCl	dl-Amidone HCl		
Dose mg/kg	Max change in respiration per cent	Dose mg/kg			Max change in respiration per cent	
1 5 1 5 2 5 2 5 2 5 2 5 2 5 5 0	+15 +24 +3 -10 +14 +12 -5 Mean +19 5 +4 7 +4 7 -5 0	1 4 1 4 1 4 1 7 1 7 1 7 2 5 2 5	-46 -32 -34 -47 -53 -53 -87 -91 Mean -37 3 -57 2 -589 0	28 28 28 28 34 34 34 35 50	Mean -55 -64 -60 -47 -66 -55 -47 -78 -94 -96 Mean -56 5 -61 5 -91 5 -95 0	

connected to a rubber mask with inspiratory and expiratory valves and fitted to unanaesthetized rabbits restrained in a prone posture by broad Since analgesic compounds comcotton tapes monly cause a marked fall in body temperature the animals were placed in a ventilated air thermostat maintained at 24° C The recorder was of the differential type described in Gaddum's paper with tambours 4 in in diameter. In these circumstances the record obtained was linearly proportional to the respiratory minute volume from a recorded height of 2 to 125 cm upon the kymograph Four to eight rabbits were used for the comparison of each compound with morphine as the "standard," and the peak percentage of respiratory depression was measured

Both dl - and l - amidone depress the rabbit respiration to a marked degree when doses are given slightly in excess of those required to produce analgesia

Table III shows the percentage change of the normal respiratory minute volume of rabbits measured at the point of maximum respiratory depression on the continuous records obtained with Gaddum's respiration recorder

The results are expressed graphically in Fig 1, from which it will be seen that the depressant effect of *l*-amidone upon the rabbit respiration is twice as great as that of the racemic compound, the effect of the *dextro* isomer being negligible in comparable doses. Some degree of stimulation is possibly caused by the latter drug

The respiratory depressant property parallels the occurrence of analgesic properties in these three compounds and is therefore not the reason for the high toxicity of all three isomers

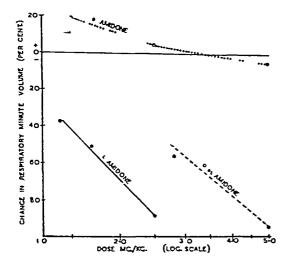


Fig 1—The effect of the optical isomers of amidone upon the respiratory minute volume of rabbits

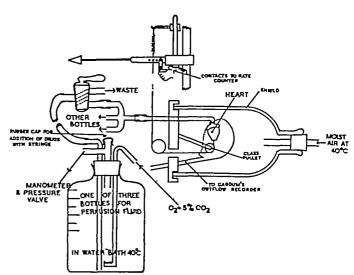


Fig 2—Apparatus for the perfusion of the isolated rabbit heart which enables the effect of accurately measured concentrations of drugs to be investigated

Effect upon the isolated rabbit heart

The effect upon the isolated rabbit heart was estimated by means of the Langendorff preparation, but a modified apparatus was used which enabled the heart to be exposed to accurately known concentrations of the drugs

This apparatus is shown diagrammatically in Fig 2 and consists of a rigid vertical board through which the aortic cannula is mounted. This cannula can be connected to any one of three bottles of 1 litre capacity by three two-way stopcocks. The bottles, filled with Ringer solution, are kept at 39° C in a water-bath By this means the drug under test can be administered in known dilution from one of the bottles, and, after the rubber tubing connecting the cannula has been flushed out by means of the two-way tap, this solution can be substituted for the Ringer solution already flowing through the heart

The outflow from the coronary vessels is recorded by means of a funnel beneath the heart carrying the perfusate to a Gaddum outflow recorder. The heart-beat is recorded by a thread from the apex of the left ventricle passing over a glass pulley and through a hole in the board to a lever on the kymograph. A record of the heart rate is made simultaneously by counting the closing of a pair of springy contacts mounted at the hinge of the recording lever. These contacts connect to a valve relay, and an impulse counter, described by the author (1948), is used to make the final record. By this means changes in coronary flow, heart rate, and the amplitude of contraction with each beat are recorded simultaneously

The data shown in Table IV were obtained by means of the method described above. Results

TABLE IV

THE EFFECT OF THE OPTICAL ISOMERS OF AMIDONE AND OTHER ANALGESIC DRUGS UPON THE LANGENDORFF
PREPARATION OF THE ISOLATED RABBIT HEART

Drug	Dilution	Effect upon the isolated rabbit heart					
Drug	Diduon	Coronary flow	Heart rate	Amplitude of beat	Notes		
d-Amidone	1 500,000	Nil	Nil	Decreased	Toxic conc		
HCl	1 100,000	+25%	Slowed	Greatly decreased			
l-Amidone	1 500,000	+20%	Nil	Decreased	Toxic cone		
HCl	1 100,000	+30%	Slight slowing	Greatly decreased			
dl-Amidone	1 500,000	+10%	Nıl	Decreased	Toxic conc		
HCl	1 100,000	+20%	Slowed	Greatly decreased			
Morphine	1 100,000	Nil	Nil	Nil	Non-toxic		
sulphate	1 50,000	Nil	Nil	Nil			
Pethidine	1 100,000 1 20,000	Nıl +10%	- Nıl Nıl	Decreased Greatly decreased	Toxic conc		

obtained with morphine and pethidine were included for comparison

It will be seen that all three isomers of amidone behaved similarly upon the heart. The toxic level was similar with each isomer and was found at concentrations of 1 100,000 and stronger. If it be assumed that the drug is distributed uniformly upon intravenous injection this level corresponds to a dose of the order of 10 mg/kg, a level comparable with that expected from the toxicity determinations

Pethidine also proved to be toxic to the heart, but the concentration required was five times as great as that of amidone isomers, this finding again agrees with the acute toxicity ratios for these drugs. Morphine, which is of low toxicity in mice and rats, was without cardiotoxic action at concentrations of 1 20,000 and less

The principal toxic action of amidone isomers is therefore upon cardiac muscle, but it is increased with the *l*- and *dl*-isomers by the central nervous depression which these two drugs produce. The apparent stimulation of respiration shown by *d*-amidone is probably real and secondary to the fall of blood pressure produced by the action upon the heart of small doses.

Spasmolytic activity

Segments of rabbit ileum, caused to contract with a concentration of 1 in 4,000,000 carbamylcholine, showed approximately 70 per cent relaxation of the spasm after the addition of any one of the three amidone isomers in a concentration of 1 in 150,000 in the isolated organ bath. When 1 in

5,000 barum chloride was used in the same way each of the amidone isomers caused similar relaxation in a concentration of 1 in 120,000

Pethidine was tested in the same way for purposes of comparison, after carbamylcholine it had a similar spasmolytic effect in a concentration of 1 in 70,000 and after barium chloride the same concentration was effective

These results show that amidone and its optical isomers are rather more active as spasmolytic drugs than pethidine. This effect is not associated with the optically active carbon atom but is a function of the molecule as a whole, the action on the isolated heart already described is equally independent of the stereochemical configuration. Probably they act directly upon the muscle cells

Local anaesthetic action

The local anaesthetic activity of the three isomers was compared with that of procaine by the intracutaneous weal method described by Bülbring and Wajda (1945) Eighteen to 24 guineapigs were used for each drug with two intracutaneous weals on each animal Each animal received one weal from procaine and one weal from one of the test compounds. In half of the group of guinea-pigs high doses of procaine or of the amidone isomer were placed anteriorly and low doses posteriorly, and in the other animals the arrangement was reversed, this was done in order to compensate for the slightly greater sensitivity of the skin in the anterior half of the body

The results were assessed by calculating the mean slope of the dose response curves for

procaine and the amidone isomers, and thereby obtaining the potency ratio for the various drugs A weighted mean potency ratio was then calculated for the group of animals treated with the same compound

TABLE V

A COMPARISON OF THE LOCAL ANAESTHETIC PROPERTIES OF THE OPTICAL ISOMERS OF AMIDONE USING THE METHOD OF BULBRING AND WAJDA

The volume of solution injected for each dose was 0 1 ml

Drug	No anımals	Conc f of drug mg/ml	Mean potency ratio (procame = 1)
d-Amidone hydrochloride	18	4 0 2 0	} 0 58
I-Amidone hydrochloride	24	2 0 1 0	} 3 51
dl-Amidone hydrochloride	24	2 0 1 0	} 2 14

The results are given in Table V from which it will be seen that local anaesthetic action is shown by all three isomers and is greatest in the *laevo* form. The potency of the racemic compound is the mean of those of the *d*- and *l*-isomers. Consequently the optical isomerism of amidone greatly influences the local anaesthetic activity, which is 3.5 times as great as procaine for the *laevo* isomer, but that this is not the only factor influencing this activity is shown by its occurrence with all three isomers.

Effect upon body temperature

The effect of these amidone isomers upon body temperature was determined in rabbits, six animals being used for each experiment. The temperatures were measured by rectal thermocouples connected to a Tinsley amplifier through a selector switch connecting each rabbit to the recorder once in 2 min. The final record was taken by means of a Siemen's six-colour chart recorder. The doses were given subcutaneously in a volume of 1 ml physiological saline.

Groups of six rabbits were used together at one time, but each drug was given to more than one such group since some rabbits occasionally showed no effect at all from the drug. This absence of effect has previously been recognized in rabbits being used for testing pyrogenic substances, and a preliminary sorting test is usually employed to select animals of comparable sensitivity. Rabbits which showed no effect with the active drugs in

these experiments were not included in the results. The remaining animals tended to fall into two groups, the larger one of which exhibited consistent changes in body temperature to the same treatment and a second smaller group of very sensitive animals which showed a very much more marked depression of temperature. These more sensitive animals numbered about 15 per cent of the total used. For the purposes of these comparisons all animals showing a fall in temperature were used for the calculation of the mean curves reproduced in Fig. 3

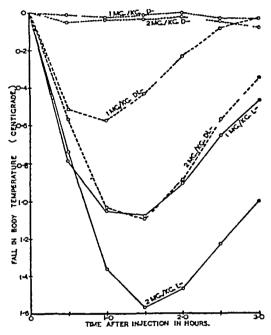


Fig 3 —The effect of optical isomers of amidone upon the body temperature of rabbits

The curves show that *d*-amidone had no effect upon body temperature, *l*-amidone produced the most marked depression, and the racemic isomer produced an effect which was very nearly half that of the *l*-isomer

DISCUSSION

The central depressant actions of amidone are associated only with the *laevo* isomer. Consequently the racemic compound is approximately half as active on the central nervous system. The effects of amidone are complicated by the fact that some structural features other than the stereochemical configuration confer on the molecule marked toxic properties to the muscle cell, since these are also displayed by the *dextro* isomer.

The action of amidone upon the cardiovascular system has been examined by Scott and his colleagues (1947) using the cross-circulation technique of Heymans in dogs. They have shown that

the direct hypotensive effect was confined to the donor dog and was not the result of vagal mediation, and they concluded, because of the slowing of the heart of the recipient dog, that amidone was a stimulant of central parasympathetic nuclei, although the drug does not show any other characteristic parasympathetic effects

The acute toxicity of amidone is almost entirely due to the direct effect on the heart whereas this type of effect is very small with morphine, this explains the findings of Finnegan et al (1948) that morphine is far less toxic in acute experiments but that in chronic experiments with smaller doses there is less difference between this drug and amidone

Compounds of the amidone type commonly show powerful local anaesthetic activity, and it is curious that among the effects which are influenced by the stereochemical configuration this was the only one in which the *dextro* momer had a considerable degree of activity

SUMMARY

- 1 The dextro, laevo, and racemic optical isomers of amidone (2-dimethylamino-4 4-diphenylheptan-5-one) have been examined pharmacologically The effects upon the central nervous system in mammals are associated with the laevo, and consequently also the racemic form
- 2 The site of action of the acute toxicity of amidone was found to be upon the cardiac muscle cells. All three isomers of amidone were approximately equally toxic

- 3 Spasmolytic activity was shown to be a function of the general structure of amidone and not associated with optical isomerism
- 4 Local anaesthetic activity occurs in all three isomers, but is influenced by optical isomerism and is greatest in the *laevo* form
- 5 The recently reported property of analgesic drugs, of producing a state of "acute vascular tolerance" to the depressor action resulting from intravenous injection, has been confirmed with *l*-amidone

My thanks are due to Messrs J B Attlesey, D R Howard, and R. F Sellar for technical assistance In addition I should like to thank Dr E Walton, of the We'lcome Chemical Research Laboratories, and Mr P Ofner, of the Wellcome Chemical Works, for the separation and provision of supplies of the optical isomers of amidone

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ANTIMITOTIC ACTION OF MALEIMIDE AND RELATED SUBSTANCES

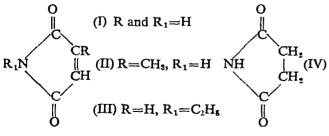
BY

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(Received October 2 1948)

The results obtained by us (1948a) with maleic acid as an inhibitor of mitosis have been developed in an attempt to prepare other mitotic inhibitors related to maleic acid, in particular we have investigated the antimitotic activity of substances in which the maleic acid residue is part of an aliphatic ring. The imides of maleic acid (I) and of citraconic acid (II), N-ethylmaleimide (III), and in addition succinimide (IV) were chosen for this purpose



The action of these substances on the growth of normal cells is reported in this paper

METHODS

The experiments were carried out on tissue cultures of chick fibroblasts. The technique adopted by us has been described previously (1948a). The hanging drop method was used in all experiments except some with maleimide, in which the Carrel flask method was adopted in combination with the Kodak record film technique.

Carrel flask technique —By this technique the tissues are grown in a solid coagulum of blood plasma at the bottom of a small flask. The embryo extract containing the compound is added as soon as the plasma has clotted

Cine micrographical record —A picture was taken every sixth minute on a Kodak recording film. The mitotic index was assessed by counting the mitoses occurring in each photograph and by making a total cell count at the end of every tenth hour. The per-

centage of hourly mitoses was calculated in terms of the number of cells present Details of this technique have been described by Willmer and Jacoby (1936)

EXPERIMENTAL

Mitotic Disturbances

The values for mitotic inhibition and phase distribution obtained with compounds I, II, III, and IV are collected in the Table

Maleimide (I, R and $R_1 = H$)

Maleimide has weak antimitotic activity At $5 \times 10^{-6}M$ an inhibition of 21 per cent was observed. By raising the concentration to $9 \times 10^{-6}M$ no further increase of the inhibition has been observed.

At lower concentrations of maleimide $1 \times 10^{\circ}$ M (Table) and occasionally at $3 \times 10^{\circ}$ M, an increase in the mitotic count occurred after 15, 3, and 24 hours (Fig 1) A number of experiments was performed to ascertain the nature of

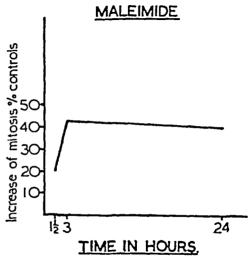


Fig 1—Increase in mitotic count with 3 × 10 ⁶M maleimide at 1 5, 3, and 24 hours

TABLE

TISSUE CULTURES CHICKEN FIÈROBLASTS, HANGING DROP METHOD, 4TH PASSAGE, 24 HOUR CULTURES, FIXED IN SUSA, STAINED IN HEIDENHAIN'S HAEMATOXYLIN

		114 30	SA, STAINED	IN REID	ENHAIN	HAEMA			<u></u> _		
Exp	Molar	Mitoses as % of mitoses of	Per cent		P	hase dist	ribution	in % of	fmitoses	1	4
	conc	controls	• I BHHIUIUUH I		hase	Meta	phase	Anaphase		Telo	phase
		M	aleimide (9,	619 mito	tic cells	investig	ated)				
1 2 3 4 5 6 7 8 9	Controls 1 × 10 ⁶ 3 × 10 ⁻⁶ Controls 5 × 10 ⁻⁶ 7 × 10 ⁻⁶ 9 × 10 ⁶ Controls 3 × 10 ⁻⁶	128 4 ± 7 9% 99 0 ± 5 4% 	0 0 21 0 23 3 21 0	15 4 12 7 16 8	14 0 19 0 22 2 16 8 28 3 17 2	47 2 39 1 30 9	34 0 25 0 29 3 30 3 28 9 22 0	6 2 5 7 3 6	68 30 21 49 49	31 2 42 5 48 7	45 2 53 6 46 4 48 0 37 9 57 4
	CITRACONIMIDE (4,634 mitotic cells investigated)										
1 2 3 4 5 6	Controls 3 × 10 ⁶ Contro s 1 × 10- ⁶ 6 × 10- ⁷ 4 × 10- ⁷	38 2 ± 2 8% 66 7 ± 2 1% 80 6 ± 3 2% 89 8 ± 4 9%	61 8 33 3 19 4 10 2	23 4 10 0	19 5 6 3 14 1 7 5	20 8 45 6	31 6 34 9 43 1 42 8	4 3 6 5	4 5 13 6 6 8 9 4	51 5 37 9	44 4 45.2 36 0 40 3
		N- е́гн	YLMALEIMIDE	(5,070 r	nitotic c	ells inve	stigated)	<u>'</u>	·	·	
1 2 3 4 5 6 7 8	Controls 4 × 10 ⁷ 6 × 10 ⁻⁷ 1 × 10 ⁶ Controls 2 × 10 ⁻⁶ 4 × 10 ⁻⁶ 6 × 10 ⁻⁶	82 0 ± 2 9% 72 7 ± 3 7% 67 9 ± 3 0% — 49 2 ± 2 4% 52 9 ± 1 9% 47 9 ± 2 3%	18 0 27 3 32 1 50 8 47 1 52 1	22 1 12 2	16 3 9 1 19 5 10 7 10 5 16 1	31 8	36 1 39 5 34 9 45 4 43 2 41 1	16	5 1 11 3 5 9 1 6 5 2 3 7	38 4	42 5 40 1 39 7 42 3 41 1 39 1
	Succinimide (5,373 mitotic cells investigated)										
1 2 3 4 5 6	Controls 3 × 10 ⁶ Controls 5 × 10 ⁻⁶ 7 × 10 ⁻⁶ 9 × 10 ⁻⁸	97 6 ± 3 5% 	2 4 	18 8 15 6	19 1 17 8 15 0 17 3	27 0 27 1	30 2 41 2 41 0 40 0	62	6 1 2 6 2 1 1 8	48 0 53 9	44 6 38 4 41 9 40 9

this increase The Carrel flask method in combination with the Kodak record film technique made it possible to count the mitoses hourly in unstained preparations, whereas in the hanging drop method stained cells are counted only after 24 hours. The film showed that some cells remained in metaphase for 8–12 hours and that other cells never completed cell division. In the 24-hour period used in the hanging drop method,

therefore, not only the cells which are going into division are counted but in addition those which persist in metaphase. Thus the Kodak record film technique gave clear evidence that the increase of the mitotic count after 24 hours consisted in an accumulation of metaphases.

Abnormal cells were found at all concentrations, they were confined mostly to clumped metaphases Enlarged and vacuolated cells were

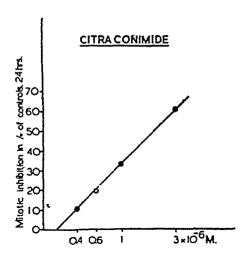


Fig 2—Mitotic inhibition plotted as percentage of controls against the logarithm of the concentration of citraconimide

present At $1 \times 10^{\circ}M$, the lowest concentration investigated, a few tripolar cells (3 in 470 metaphases), fragmented chromosomes, and chromosome bridges (10 in 142 anaphases) were observed

Citraconimide (II, $R = CH_1$, $R_1 = H$)

Citraconimide had the strongest antimitotic properties of the substances of this group. The mitotic inhibition increased with rising concentration. At $3 \times 10^{\circ}M$ the inhibition was 61.8 per cent. The increase followed closely a logarithmic graph when plotted against the \log_{10} of the concentrations (Fig. 2). Microscopically no abnormalities were found in significant quantities

N-ethylmaleimide (III, R = H, $R_1 = C_2H_5$)

N-ethylmaleimide showed increasing antimitotic activity from $0.4 \times 10^6 M$ to $2 \times 10^6 M$, at the latter concentration 50.8 per cent inhibition was observed. A further rise in concentration did not lead to greater inhibition. Microscopically, no abnormalities in significant numbers are present

Succinimide (IV)

Succinimide showed no mitotic inhibition and no abnormal cells were observed

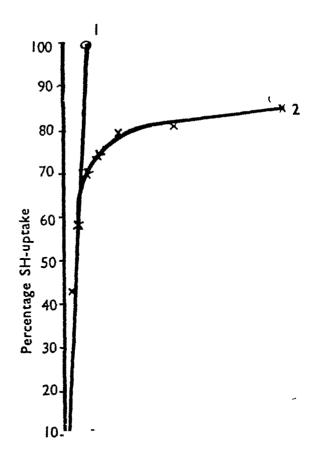
Sulphydryl Uptake

In a previous paper (1948b) we drew attention to a parallelism between mitotic inhibition and -SH uptake, which was clearly evident in the maleic acid series. In order to ascertain whether a similar parallelism could be established in the maleimide series, we investigated the -SH uptake of maleimide, citraconimide, and of N-ethylmale-

imide, using thiolacetic acid and glutathione as -SH donors and following the directions given by Morgan and Friedmann (1938) for maleic acid and these two -SH compounds

At room temperature and at a final concentration of M/50 for each component, maleimide took up 100 per cent of thiolacetic acid and 100 per cent of glutathione within 2 min. Citraconimide took up 70 per cent of thiolacetic acid and 75 per cent of glutathione within 3 min. After this quick uptake the reaction slowed down and the curves representing it become asymptotic N-ethylmaleimide behaved like maleimide and showed 100 per cent -SH uptake from thiolacetic acid and glutathione within 1 min.

Fig 3 demonstrates the -SH uptake from glutathione as -SH donor with maleimide and citraconimide as -SH acceptors at room temperature



2 4 6 8 10 12 14 16 18 20 TIME (MIN)

Fig 3 —SH uptake of maleimide and citraconimide Curve 1 maleimide and glutathione, final concentration M/50 Curve 2 citraconimide and glutathione, final concentration M/50

DISCUSSION

Of the four substances investigated, three are unsaturated and only one, succinimide, is saturated The three unsaturated substances, maleimide. citraconimide, and N-ethylmaleimide, have antimitotic properties, whereas the saturated compound, succinimide, is devoid of antimitotic These mitotic inhibitors share with the other mitotic inhibitors, encountered previouslyie, maleic acid and the 1 4-naphthoguinones --- the property of forming -SH adducts.

The -SH uptake of the maleimides is extremely rapid compared with the -SH addition to maleic acid M/25 maleic acid needs 6 hours for a 50 per cent -SH uptake from M/50 thiolacetic acid and M/50 glutathione, whereas maleimide and Nethylmaleimide accomplish a 100 per cent -SH uptake from the same substances in 1-2 min and citraconimide a corresponding uptake of 70 per cent in 3 min. It will be seen that the introduction of a methyl group in maleimide decreases the -SH uptake as shown by citraconimide

The mitotic inhibition of the imides exhibits a picture which is very different from the mitotic inhibition observed in the maleic acid group Maleimide is less active than maleic acid, whereas citraconimide is quite active, although the free acid was inactive. The readiness of the maleimides to add sulphydryl compounds and the presence of glutathione in the medium used for the tissue cultures (Berger and Peters, 1933) may show the way for a reasonable explanation of these results Experiments in this direction are in progress

SUMMARY

- The antimitotic activity of maleimide (I). citraconimide (II), N-ethylmaleimide (III), and of succinimide (IV) has been tested in tissue cultures of chick fibroblast The unsaturated imides (I, II, III) were active in the concentration range of 10 M The highest activity was shown by III, the methyl derivative of maleimide The saturated imide (IV) was inactive
- 2 The -SH uptake of the unsaturated imides has been determined The introduction of a methyl group in maleimide decreases its reactivity towards -SH compounds

One of us (EF) is indebted to Messrs May and Baker, Ltd Dagenham, Essex, for financial support

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"CONSTANT FLOW" ORGAN-BATH TECHNIQUES

BY

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(Received October 27, 1948)

It has remained the common practice, since Magnus (1904) first showed that an excised strip of intestine would continue its rhythmic contractions if suspended in warm oxygenated Ringer-Locke solution, to change the contents of an organ bath by a series of washings A drug is injected to give a known final concentration, rapid mixing being brought about by the stream of oxygen bubbles passing up through the bath, then, when the response of the muscle has been recorded, the solution is flushed out and replaced with fresh Ringer-Locke solution Good techniques of this kind have been described recently by Chen, Ensor, and Clarke (1948) and by Miller, Becker, and Tainter (1948)

That it might be advantageous for some purposes to change the contents of an organ bath gradually instead of intermittently occurred to us in the course of experiments in which blood vessels were perfused at a constant rate. These studies (Fastier, 1948, Fastier and Reid, 1948) suggested that the duration of pharmacological effects observed under such conditions might be no less significant than their initial magnitude, for when a common site of action is indicated, the more active of two stable drugs is likely to be the one whose structural features, apart from providing the necessary "active groups," permit stronger adsorption at the site of action (Albert, 1944, Pfeiffer, 1948) It seemed important to check the idea that at least some of the drugs which have lasting effects upon preparations of this type are ones which are retained tenaciously by the muscle in the face of constant washing with the perfusing salt solution Accordingly we sought a technique which would enable the outflowing Ringer's solution to be collected for analysis at the same time as the pharmacological effects of an added drug were being recorded

The first type of apparatus devised for the purpose utilized a constant output pump (Messrs Palmers' model F31), and the most compact form is illustrated Well-oxygenated Ringer's solution is pumped from the reservoir R into the thermostatically controlled vessel V, which in turn maintains a constant temperature in the small organ bath B Ringer's solution is forced into the bath B at a constant rate via the rubber tubing I, which also provides a convenient site for the injection of drug solutions We have found that even when the pendulum movement of a muscle strip is quite vigorous, reasonably constant changes in tonus can be obtained by injecting such drugs as adrenaline, histamine, and acetylcholine in fixed small doses (Fig 2) A good base-line is provided meanwhile by the general level of tonus The Ringer's solution finally escapes through the overflow O, where successive portions may be collected for analysis

The arrangement shown in Fig 1A does not require a mechanical pump, a steady flow of Ringer's solution

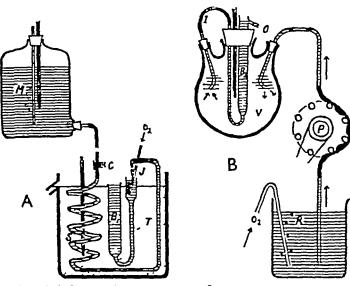


Fig. 1 (schematic) —Two types of apparatus permitting a slow, steady flow of Ringer's solution through organ baths $(B_1 \text{ and } B_2)$

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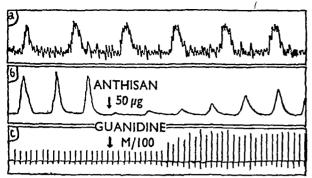


FIG 2—Facsimiles of kymograph records obtained by means of constant flow organ-bath techniques

(a) Rabbit ileum washed with Ringer-Locke solution The increases in tonus were produced with 0.2 μg doses of acetylcholine injected at 3-min intervals Pumping rate 30 c c per min
 (b) Guinea-pig ileum washed with Krebs-Hense-

(b) Guinea-pig ileum washed with Krebs-Henseleit solution Histamine (5 μ g) injected at 4-min intervals "Anthisan" (50 μ g) injected at the arrow 1 min before the fourth injection of histamine

(c) Frog sartorius stimulated electrically at 10-sec intervals. Guanidine hydrochloride (M/100) added to the perfusing Ringer-Harvey solution at the arrow. The effect of the stimulus is soon potentiated.

being secured by means of the Mariotte bottle M. The screw-clamp C adjusts the output. After passing through the spiral S in the thermostatic bath T, the Ringer's solution drips into the receiving chamber J where it may be further oxygenated before it reaches the preparation. Drugs are injected here. In order to decide upon the best shape for the bath it has been found helpful to watch how a small quantity of injected dyestuff streams past the preparation, this shows whether a drug solution will be distributed satisfactorily in transit

To what extent the various principles followed—constant flow of Ringer's solution, "external' oxygenation and addition of drugs, the strip filling up most of the bath and always immersed in

Ringer's solution—have been used previously, has proved almost impossible to ascertain. This is due not only to the widespread use of organ-bath technique but also to the relative infrequency with which it has been described on its own account. In a paper on the assay of substances liberated from adrenergic nerves, Gaddum, Jang, and Kwiatkowski (1939) have shown that it is very convenient to drip the perfusate from the organ under investigation on to a strip of hen's rectal Later Kwiatkowski (1941) successfully modified this dripping technique for the assay of More recently Schild (1947) has wellnigh perfected a method based upon the removal by suction of the excess of Ringer's solution entering a bath from a reservoir, he has also demonstrated the advantages of adding an antagonist drug to the bath fluid before it reaches the gut

SUMMARY

It has been found advantageous for some purposes to change the contents of an organ bath gradually instead of intermittently. Two types of apparatus based upon this principle are described

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THE MECHANISM OF ACTION OF ANTICHOLINESTERASE DRUGS

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During the past few years much new work on anticholinesterase substances has been published as a result of the new interest in the subject produced by the discovery of disopropylfluorophosphonate (DFP) by McCombie and Saunders (1946) Many discrepancies of detail between the behaviour of individual cholinesterase inhibitors have been noted and have occasioned serious doubts as to the validity of the hypothesis that the physiological action of these substances is solely a consequence of cholinesterase inhibition One source of error was greatly clarified by Mendel and his co-workers (Mendel, Mundell, and Rudney, 1943, Mendel and Rudney, 1943 and 1944. Hawkins and Gunter, 1946. Hawkins and Mendel, 1947), who showed that the term "cholinesterase" has been applied in the past to enzymes of two, and possibly more (Augustinsson, 1948), distinct enzymological species—termed by them "true" and "pseudo" cholinesterases, distinguishable by specific substrates They have shown that these enzyme types differ strikingly in their distribution, their sensitivity to inhibitory agents, and their optimum substrate concentrations, and that the appearance of the pharmacological effects of anticholinesterases is related to inhibition of the "true" cholinesterase Only when the physiological response measured is that to acetylcholine carried by the blood stream does the pseudocholinesterase in the plasma seem to be important (Heymans, Verbeke, and Votava, 1948) in determining the magnitude of acetylcholine Despite this very important advance many anomalies remain to be explained, and it is the purpose of this paper to discuss the kinetics of cholinesterase inhibition by various agents and the light this sheds on our interpretation of cholinesterase inhibition under strictly physiological conditions Some aspects of the kinetics of cholinesterase activity and inhibition have been considered by a number of workers (Straus and Goldstein, 1943, Goldstein, 1944, Mazur and

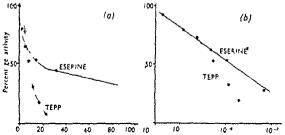
Bodansky, 1946, Nachmansohn, Rothenburg, and Feld, 1947, Augustinsson, 1948, Brauer, 1948, Jansen, Nutting, and Balls, 1948, Mackworth and Webb, 1948, Nachmansohn, 1948), but there has been little attempt to interpret these results in terms of prevailing physiological circumstances, and in consequence the design of experiments on cholinesterase activity has usually been inadequate to provide relevant data. In the account that follows attention will be concentrated on the contrast between the modes of action of the two main groups of anticholinesterases, typified by eserine and tetraethyl pyrophosphate, with notes on such differences from these patterns as are found with other inhibitors

MATERIALS AND METHODS

The enzyme preparations used were, (1) fresh oxalate human plasma which contains predominantly pseudocholinesterase, (2) washed human red blood cells, lysed by the addition of 4 volumes of 0 025M NaHCO_a, containing only true cholinesterase, (3) in some experiments a highly purified bovine red cell true cholinesterase. obtained from Dr M L Tainter, has been used each mg of which could hydrolyse 3 mg of d(+) acetyl- β methylcholine chloride per minute The activity of the enzyme was estimated in the Warburg manometric apparatus with 0 025M NaHCO, as medium equilibrated with 95 per cent $N_2 + 5$ per cent CO_2 gas mixture at 37° C The enzyme solution was normally placed in the main compartment of the vessel, and the substrate and inhibitor, each dissolved in 0.2 ml of bicarbonate solution, were placed in separate side arms. The total volume of fluid used was always 3 ml The usual final concentration of substrates were 0 025M dl-acetyl-\(\beta\)methylcholine chloride, 0 007M benzoylcholine chloride, and 0.02M acetylcholine chloride. Where other concentrations of substrate have been employed they are mentioned in the text. In all experiments corrections for non-enzymic hydrolysis of the substrate were applied Dilute solutions of the enzyme inhibitors have been made immediately before use from stock solutions, or with tetraethylpyrophosphate (TEPP) and disopropylfluorophosphonate (DFP) from the pure substances

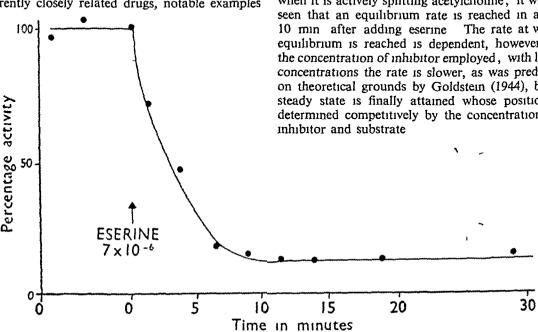
RESULTS

There is a tendency in pharmacology to assume an identical mechanism for two drugs that have similar actions if their dose-response relationships are similar. In Fig. 1 data for eserine and TEPP inhibition of true cholinesterase are given from the curve for TEPP being much steeper than



1 -Human red cell cholmesterase Substrate 0 025M acetyl-\(\beta\)-methyl choline chloride Incubation of inhibitor and enzyme for 20 min before addition of substrate Ordinates velocity of hydrolysis of substrate as percentage of uninhibited control rate Abscissae concentration of inhibitor in g/ml (a) Arithmetical scale, (b) log scale

that for eserine and consequently appearing nearly linear over the range 0-70 per cent inhibition, there is nothing in such data to suggest the striking difference in kinetics of action that will be discussed Such differences of dose-response slope are very commonly encountered in pharmacology among apparently closely related drugs, notable examples



2—Human red cell cholinesterase. Substrate 0.02M acetylcholine chloride. At zero time addition of 7×10^{-6} g eserine sulphate/ml. Ordinates percentage of activity before addition of inhibitor. Abscissae time in min after addition of inhibitor

being found in the central analgesic and local anaesthetic series, without arousing any doubts as to a unitary mechanism of action. It should be noted that this steep dose-response curve may account for a feature of TEPP action-namely, that its pharmacologically effective dose is much closer to the lethal dose than is the case with eserine or prostigmine

Kinetics of Eserine Action

(a) Rate of combination of eserine and enzyme in the absence of substrate

At present it is not possible to measure this reaction directly, it can only be inferred from indirect data. If eserine is left in contact with the enzyme for 1, 5, or 20 min at 37° C before addition of substrate the subsequent rates of hydrolysis are indistinguishable, but such information is not helpful. The rate of combination in the absence of substrate-1e, under non-competitive conditions-however, must be faster than in the presence of substrate. It will be seen in the next section that under these conditions combination occurs fairly rapidly

(b) Rate of combination of eserine and enzyme in the presence of substrate

Fig 2 shows some typical results illustrating the rapidity with which eserine reacts with cholinesterase when it is actively splitting acetylcholine, it will be seen that an equilibrium rate is reached in about The rate at which equilibrium is reached is dependent, however, on the concentration of inhibitor employed, with lower concentrations the rate is slower, as was predicted on theoretical grounds by Goldstein (1944), but a steady state is finally attained whose position is determined competitively by the concentrations of

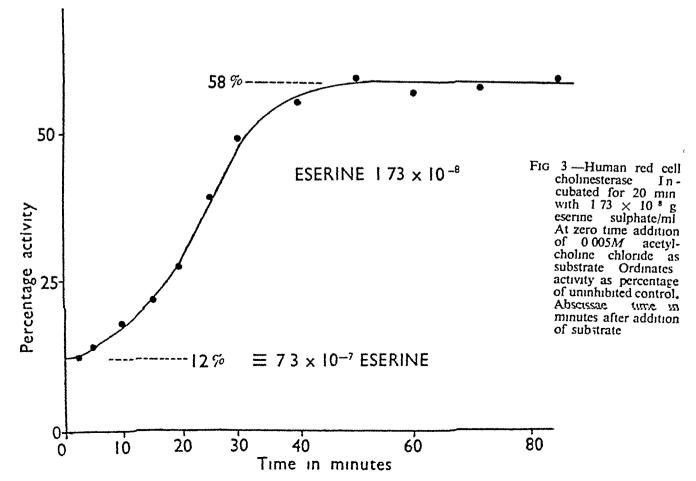
(c) Rate of dissociation of eserine-enzyme complex in presence of substrate

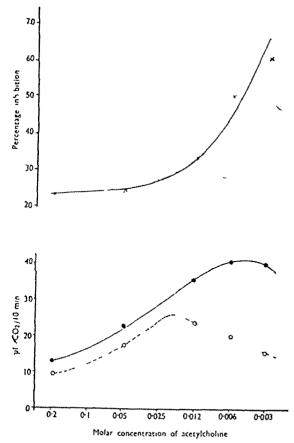
When substrate is added to a mixture of enzyme and inhibitor the enzyme-inhibitor complex is decomposed according to the Michaelis-Menten equilibrium as follows

$$EI \rightleftharpoons E + I$$
 (1)
 $E + S \rightleftharpoons ES$ (11)

where E = free enzyme concentration, I = free inhibitor concentration, S = free substrate concentration. EI = inhibitor-enzyme complex, ES =enzyme\substrate complex, and the velocity of acetylcholine hydrolysis is dependent on the concentration of ES Fig 3 illustrates an experiment designed to examine the velocity of this change The initial measured rate of hydrolysis was less than 12 per cent of an uninhibited control, and the shape of the curve would suggest that under these experimental conditions the control value was probably not much less than this, but it has not been found possible to obtain reliable figures in the first 1-2 minutes owing to insufficient lapse of time for the attainment of temperature equilibrium. Over the succeeding 50 min the rate gradually rose until it reached an equilibrium rate which was 58 per cent of the control. The rate of attainment of equilibrium is dependent on the concentration of inhibitor and substrate employed—in particular low substrate concentrations prolong the period to equilibration and high substrate concentrations speed it up. The initial value obtained is, however, virtually independent of substrate concentration and is presumably dependent purely on the position of equilibrium in equation (i) before substrate competition has appreciably shifted it. The final equilibrium level of activity reached is, however, influenced by both inhibitor and substrate competitively

It is clear that values obtained for cholinesterase inhibition will vary according to the arbitrary conditions selected Equilibrium values will depend both on substrate concentration and the substrate employed. The higher the substrate concentration (Fig. 4) the smaller the degree of inhibition d-Acetyl-β-methylcholine, which is employed as a specific substrate for "true" cholinesterase, has a lower affinity for the enzyme than acetylcholine, and in consequence eserine shows a greater equilibrium inhibition of cholinesterase with acetyl-β-methylcholine than with acetylcholine as substrate





4 -- Human red cell cholmesterase Substrate acetylcholine chloride Lower curve Ordinates velocity of acetylcholine hydrolysis in µl CO₂ evolved/10 min at equilibrium Abscissae molarity of acetylcholine • -● without addi-O---O with 1.7×10^{-7} g tion of eserine eserine sulphate/ml Upper curve Ordinates percentage inhibition of cholinesterase by eserine Abscissae molarity of acetylcholine

If, however, other than equilibrium conditions are considered the situation becomes chaotic. Many authors have reported results obtained in the first twenty or thirty minutes after adding substrate, but these data are influenced in such a complex way by both inhibitor and substrate concentration that their quantitative value is very doubtful. It would be of value to be able to determine the degree of non-competitive inhibition since this is unaffected by substrate concentration, but the experimental difficulties make the values only approximate, as they are necessarily obtained by extrapolation

It is important to correlate these *in vitro* data with physiological events. The view that acetylcholine acts as a synaptic transmitter, accepted by most workers, requires that when no impulse is being transmitted at the synapse either no or an

extremely small amount of acetylcholine leaks out of the cholinergic nerve terminals, whereas with the passage of an impulse a relatively large amount of acetylcholine is liberated and is present at the synaptic region for a period of milliseconds means that in the quiescent synapse we have essentially non-competitive conditions for cholinesterase inhibition. When acetylcholine liberation occurs its persistence is very brief compared with the long period required to establish competitive equilibrium. and is certainly insufficient to allow for more than a very small displacement of the inhibitor to occur It would seem, therefore, that the equilibrium value discussed above grossly underestimates the effect of eserine under these conditions. The data in Fig. 3 show that the concentration of eserine employed. (1.7×10^{-8}) produced only 42 per cent inhibition of the enzyme at equilibrium but produced at least per cent inhibition under non-competitive To produce 88-per cent inhibition at equilibrium would require more than forty times as much eserine as was added A further factor may come into play when the enzyme is greatly inhibited, since owing to the decreased rate of hydrolysis the acetylcholine concentration may rise sufficiently to cause some competitive decrease of the inhibition In a prolonged nerve tetanus this displacement will increase during the course of the tetanus as acetylcholine accumulates and will reach an equilibrium value if the tetanus is of sufficient duration, but the position that this equilibrium is likely to reach cannot be estimated owing to our complete ignorance of the effective acetylcholine concentrations attained at the sites of cholinesterase activity in the synapse

Another important aspect of these fast reactions has never been considered in connexion with cholinesterase, and that is the biphasic character of enzymic hydrolysis required by the Michaelis-Menten theory The reaction occurs in two stages

$$E + Ac Ch \rightleftharpoons (E Ac Ch)$$
 (1)
 $\angle (E \cdot Ac Ch) \rightleftharpoons E + Ac + Ch$ (2)

Stage (1) effectively removes acetylcholine without breaking it down and hence is a reaction of primary importance in disposing of acetylcholine. It is obvious that reaction (1) occurs in a shorter time than the overall reaction (1+2), and it is therefore of fundamental importance to know the relative rates of reactions 1 and 2. So far it has not been feasible to do this with cholinesterase, but we know from the direct measurements of Britton Chance (1943, 1948) on peroxidase and catalase that the first reaction $(E+S\rightleftharpoons ES)$ may be as much as a hundred times faster than the second reaction and is mainly limited by probability considerations. If

this state of affairs can be applied to cholinesterase, it may be that the effective removal of part of the liberated acetylcholine can occur extremely rapidly That this may be so is supported by the recent work of Eccles and MacFarlane (1949) on the effect of anticholinesterases on the frog end plate potential They found that the upstroke of the end plate potential, which occurred in about 2 msec, rose higher and more steeply in the presence of anticholinesterases Considering the low temperature at which they were working (16-18° C) this time makes it probable that normally the formation of the enzyme-substrate complex is the most important factor in the initial rapid removal of acetylcholine, and that providing the enzyme capacity is adequate the actual hydrolysis of the complex is less important Anticholinesterases will, of course, inhibit this reaction in a non-competitive manner

Neostigmine behaves in a very similar way to eserine in all the equilibria so far considered, but other reversible inhibitors may behave differently. For instance, "62C47" (bis-trimethyl-aminophenylethyl ketone diiodide, Glock and Mogey, 1948) reaches equilibrium more rapidly than eserine or neostigmine, so that the equilibrium under physiological conditions may be more competitive than with eserine or neostigmine 62C47 is a less active inhibitor than eserine or neostigmine, and, as Goldstein (1944) has shown, the mass action velocities require that the less active a reversible inhibitor the more rapidly it should come into equilibrium, as is found experimentally

Kinetics of TEPP Action

(a) Rate of combination with the enzyme in the absence of substrate

It is very easy to measure the rate of combination of TEPP with cholinesterase, because, as will be seen later, not only is the reaction almost completely irreversible in vitro, but the progress of the reaction is effectively blocked by addition of substrate When cholinesterase was incubated with TEPP the enzyme slowly decreased in activity (Fig 5) so that even after 40 min the reaction was still proceeding, but at 2 hours little further inactivation was By this time the amount of available TEPP must have fallen considerably as a result of hydrolysis, and this side reaction militates against the continued progress of the reaction The initial rate of the reaction fits reasonably well the requirements for a bimolecular reaction Thus if TEPP is added to the enzyme before addition of substrate it is evident that the degree of enzyme inhibition found is entirely dependent on the time of contact allowed before addition of substrate This is in marked

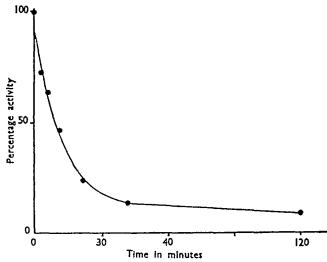


Fig 5—Human red cell cholinesterase Substrate 0.025M acetyl- β -methylcholine chloride Incubation of 8×10^{-9} g tetraethyl pyrophosphate/ml with enzyme before addition of substrate Ordinates activity as percentage of control Abscissae period of incubation of inhibitor with enzyme before addition of substrate

contrast to the state of affairs with eserine experiment recorded in Fig 1 20 min contact between enzyme and TEPP was allowed. choice of this time was arbitrary, but it is evident that for results to be comparable the same time interval must always be used Actually, providing at least 10 min contact is allowed, the error in estimating the potency of TEPP will be relatively small owing to the very steep concentrationinhibition relationship characteristic of this substance It should be noted that DFP behaves very much in the same way as TEPP on the enzyme, but, as has also been found by Mackworth and Webb (1948), combines rather more slowly

(b) Combination of TEPP with enzyme in the presence of substrate

If the experiment illustrated in Fig 2 is repeated with TEPP as the inhibitor no detectable inhibition of cholinesterase results in the succeeding hour (Fig 6, III), and even if TEPP and the substrate are added together very little inhibition results This blocking by substrate is seen when either acetylcholine or acetyl-\beta-methylcholine is used as substrate for true cholinesterase Even if the concentration of TEPP is increased to 10-100 times that usually required to produce inhibition, either no or only slowly developing inhibition occurs With DFP and some other members of the group this is by no means so, for instance, with DFP some inhibition may gradually become apparent 6, IV) even with concentrations (0.02-0 08 μg./ml) producing considerable inhibition in

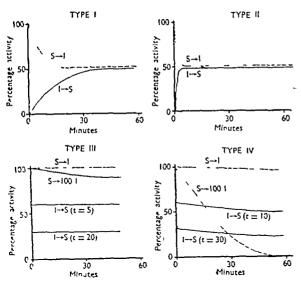


FIG 6—Diagrammatic representation of the four main classes of cholinesterase inhibitors studied. Ordinates percentage activity of enzyme Abscissae time in minutes $S \rightarrow I$ means substrate added before addition of inhibitor $S \rightarrow 100$ I substrate added before 100 times the amount of inhibitor needed to produce adequate inhibition in absence of substrate $I \rightarrow S$ inhibitor added before substrate. The time of contact in minutes between inhibitor and enzyme, before addition of substrate, is shown in parentheses $I = \text{typical results obtained eserine, neostigmine, and 'Nu683'', II = results with "62C47'' III = with TEPP IV = with DFP$

the absence of substrate, and with higher concentrations (2–8 μg /ml) inhibition appears quite rapidly despite the presence of substrate

This blocking of TEPP inhibition by substrate may have some physiological significance. It may mean that at an active synapse the presence of acetylcholine may hinder the inhibitory effect of TEPP, so that the cholinesterase at that synapse is less inhibited than at a quiescent synapse. This blocking action of substrate for TEPP inhibition has been demonstrated clearly on the frog rectus abdominis preparation by Hobbiger (1949)

Blocking of TEPP inhibition by other inhibitors

The blocking action of substrate on TEPP is presumably due to competition for the same active groups on the enzyme, and it was therefore of interest to see whether cholinesterase inhibitors, which have a far greater affinity for cholinesterase than acetylcholine, would be able to block these groups as well Fig 7 illustrates a typical experiment of this kind TEPP itself in a concentration of 1 33 × 10-8, when incubated with the enzyme for 20 min, produced a 94 per cent inhibition

Curve b shows the inhibition (Fig. 7, curve a) produced by increasing concentrations of eserine alone, whilst curve c shows the effect of incubating the enzyme with eserine for 20 min followed by incubation for 20 min with TEPP. It will be seen that at all concentrations of eserue some degree of protection of the enzyme results which increases with the concentration of eserine Curve d is an expression of the percentage protection of the enzyme from inhibition by TEPP as a result of the prior contact of the enzyme with increasing concentrations of eserine, at a concentration of 10-8 eserine protects the enzyme to the extent of about 90 per cent from TEPP inhibition It was also found that neostigmine, Nu 683, 62C47, carbachol, and choline were more or less efficient in blocking the action of TEPP, but that NaF, which by itself produces a 50 per cent inhibition of the enzyme at about 10-3 M. did not hinder TEPP inhibition and was in fact additive to it It is easy to test rapidly whether a cholinesterase inhibitor blocks the action of TEPP by using a concentration of the reversible inhibitor that produces about 50 per cent inhibition and a concentration of TEPP that will produce 95-98 per cent inhibition in 20 min. If the substance is active as a protective agent the total inhibition will lie between 50 and 90 per cent. Koelle (1946) also showed that eserine, neostigmine, and to some extent carbachol could protect cholinesterase from irreversible inhibition by DFP He was unable to show protection by acetylcholine or acetyl-8-methylcholine with his technique, which consisted of exposure of the enzyme to the protecting agent, followed by exposure to DFP for 30 minutes and subsequent dialysis against running water His figures for inhibition of enzyme before dialysis, however, show clearly the effect of substrate described above Koster (1946) has also shown that eserine has a protective action against DFP poisoning in vivo, and this has been confirmed by others

The blocking of inhibitor action by substrate throws considerable light on the mechanism of the anticholinesterase action of the alkyl phosphate group of inhibitors These agents might act in three ways (a) a chemical reaction might occur between enzyme and inhibitor involving inactivation of both, (b) a chemical reaction might occur in which the effect of the inhibitor was catalytic and only the enzyme was changed, or (c) an initial reversible physical adsorption of inhibitor on the enzyme might occur followed by either (a) or (b) Nachmansohn, Rothenburg, and Feld (1947) have presented evidence that the inhibition of cholinesterase by DFP is biphasic, with an early reversible The data just phase and a later irreversible phase presented support and extend this view

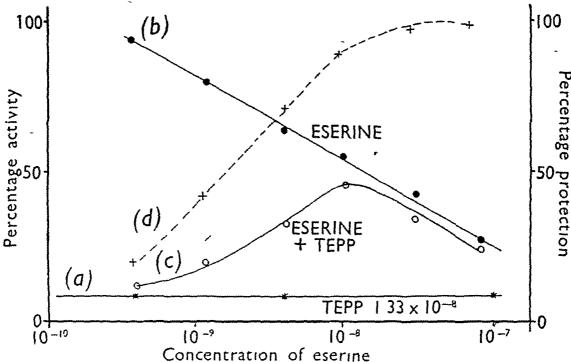


Fig 7—Human red cell cholinesterase Substrate 0.03M acetyl-β-methylcholine chloride Ordinates (left) percentage activity of enzyme Abscissae concentration of added eserine sulphate Curve (a) activity after incubation for 20 min with 1.33 × 10-8 g TEPP/ml (b) activity after incubation for 20 min with eserine sulphate Equilibrium velocities (c) incubation with eserine sulphate for 20 min followed by 1.33 × 10-8 g TEPP/ml for 20 min Equilibrium velocities (d) percentage protection by eserine of the cholinesterase from inactivation by TEPP (right-hand ordinates)

blocking action of substrate is presumably due to the inability of TEPP to react with other than free enzyme centres According to Michaelis and Menten (1913) the amount of free enzyme available is governed by the adsorption equilibrium

$$E + S \rightleftharpoons ES$$

For a given substrate concentration the proportion of enzyme molecules uncombined (E) at any instant is fixed, and for the substrate concentrations used in our experiments is only a small proportion of the total available enzyme. If the rate of enzyme inactivation by alkyl phosphates depends entirely on the number of free enzyme centres, blocking by substrate should occur equally against DFP and TEPP, but this is not so. If, however, a reversible competitive combination is a preliminary to irreversible mactivation a second equilibrium will be involved.

$$E + I \rightleftharpoons EI$$

and the rate of inactivation of enzyme in the presence of substrate will be influenced by the dissociation constant of EI which will determine the amount of EI formed in the overall competitive reaction

$$ES + I \rightleftharpoons EI + S$$

and just as with purely reversible inhibitors, a small dissociation constant for EI will favour its formation and a minimal blocking action by substrate, whereas if the dissociation constant is large—i e, the affinity of enzyme for inhibitor is small—the blocking action by substrate will be considerable The different ease of blocking with substrate can be explained if TEPP has a low adsorption affinity whereas that of DFP is high. The potency of an alkyl phosphate inhibitor thus depends on (a) the adsorption affinity, (b) the rapidity with which irreversible inactivation is produced in the enzyme-inhibitor adsorption complex. It remains to be established whether the second process is catalytic or involves the disappearance of inhibitor Brauer (1948) has indicated that TEPP becomes no longer available when it is treated with cholinesterase. The quantitative nature of this change has been investigated as follows

A constant amount of TEPP (final concentration 2.5×10^{-8}) was incubated with 0.02-0.5 mg of purified cholinesterase per ml at pH 7.2 and 37° C for 30 min controls were simultaneously run containing either cholinesterase alone or 0.5, 1.0, and 2.5×10^{-8} TEPP alone. After 30 min the pH was brought to 3.5 to 4.0 and the solution heated

at 96° C for 3 min This procedure has been found to destroy the residual cholinesterase activity completely whilst causing only very slight breakdown The solutions were then cooled and the of TEPP pH adjusted to 72 One ml of each solution was then incubated with 0 02 mg of cholinesterase for 30 min and the resultant activity of this indicator The results obtained are enzyme determined. illustrated in Fig. 8 (a), which shows that the more cholinesterase present initially the less the inhibition of the indicator enzyme. In order to determine whether these results could have been due merely to increasing protein concentration, the latter was held constant by adding to each tube a large excess of crystalline serum albumin, this procedure made no difference to the values obtained Further evidence of the specificity of the reaction was obtained by the failure of either albumin alone or heat-denatured cholinesterase to decrease the available TEPP These results fully confirm the experiments of

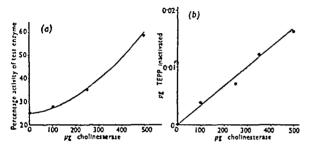


Fig 8 —Purified bovine red cell cholinesterase Substrate 0 025M acetylcholine chloride (a) Ordinates percentage activity of test enzyme Absenseae added cholinesterase initially (experiment (b) Ordinates described in text) TEPP μg inactivated Abscissae μg cholmesterase added initially

Brauer (1948) Fig 8 (b) relates the amount of cholinesterase added in the experiment of Fig 8 (a) to the amount of TEPP that has become unavailable as estimated by inhibition of the indicator enzyme. It will be seen that the relationship is approximately linear, 1 mg of cholinesterase removing about 0.034 μ g of TEPP under these conditions. A purely catalytic role of TEPP in the cholinesterase inactivation is thus excluded, and it is interesting to note that when the enzyme is heat-acid-denatured it loses its ability to combine with the alkyl phosphates as well as its enzymatic activity, and this argues further for the specificity of the chemical reaction involved.

If the biphasic interpretation of the action of the alkyl phosphates is correct, what is the nature of

the secondary reaction? All the active members of the series contain the grouping



where the groups R and R' may be a variety of structures and still retain activity, although the most active compounds are those in which R and R' contain short alkyl chains X is always a potentially acidic radicle so that the bond (a) is analogous to that in an acyl anhydride or halide and consequently rather less stable than the alkoxy or amido bonds joining R and R' to the phosphorus In view of this basic unit the most likely chemical change is a phosphorylation of the enzyme transferring the



radical to some polar grouping on the enzyme

Most of the really active inhibitors are only weak phosphorylating agents, and in general increasing reactivity of the bond (a) decreases anticholinesterase activity, thus disopropylchlorophosphonate is a much more active phosphorylator than dissopropylfluorophosphonate and yet is far less active as a cholinesterase inhibitor, in the series dimethyl-, diethyl-, and disopropyl-fluorophosphonates the reactivity of bond (a), assessed by ease of hydrolysis, decreases in that order, yet the anticholinesterase activity increases in the same direction (Mackworth and Webb, 1948, Mazur, 1946) This is not always true, for instance, in the series of tetra-alkyl pyrophosphate derivatives in which one or both (P = O) groups are replaced by (P = S) the chemical reactivity falls as well as the anticholinesterase acitivity It would appear that there is an optimum reactivity in the bond (a) which may be explained in the following way Provided that the adsorptive affinity for the enzyme remains unchanged, if bond (a) is made more reactive the rate of reaction with the enzyme will be increased, but the rate of nonspecific actions such as phosphorylation of random amino, hydroxyl, or phenolic groups in the protein may also increase, as will hydrolysis by water molecules These side reactions will divert some of the active agent-an important matter with substances effective at very low concentrations order to explain the selectivity of the alkyl phosphates it is reasonable to assume that the adsorption complex of enzyme and alkyl phosphate introduces strain in bond (a) and thus facilitates phosphorylation by these weak acylating agents

There are two alternative chemical reactions that may be considered briefly. The group X may combine with the enzyme, this is improbable because of the variety of chemical groups that are active in this part of the molecule, examples are

These groups differ greatly in chemical properties, and a mechanism which involves that they should all be highly active is clearly less probable than the relatively homogeneous mechanism suggested here It is just possible that an alkylation is involved

but these bonds are very stable to acid and alkaline hydrolysis, unlike bond (a) Further R and R' may be alkoxy or dialkylamino without a very large change in activity and yet the groups to be transferred are vastly different in chemical potentialities, finally dialkoxy-, alkyl-, or aryl- phosphonates which have similar ester bonds but no anhydride structure are quite inert as cholinesterase inhibitors

Brauer (1948) rejected the phosphorylation hypothesis on the basis of experiments with HETP (a mixture of alkyl phosphates obtained by heating ethyl phosphate with P2Os or POCl3, whose main active constituent is TEPP) labelled with P32, in which he was unable to demonstrate association of the labelled phosphorus with the enzyme His data, however, show that his preparation of labelled HETP contained only about 0.1 per cent of material active against cholinesterase, the association of this small amount of material with the enzyme would not be detectable under his experimental conditions Michel and Krop (1949) have recently carried out a model experiment of this kind using DFP labelled with P32 and electrophorus cholinesterase found that the P32 of labelled DFP was precipitated with the cholinesterase by trichloracetic acid and the amount precipitated was proportional to the amount of cholinesterase added and to the degree of cholinesterase inhibition, 1 mg of completely inactivated cholinesterase contained the phosphorus from 0.1 µg DFP, and when allowance is made for the difference of enzyme activity and the molecular weights of DFP and TEPP this figure is very close to the one we obtained for TEPP and red cell cholinesterase by the indirect method It can be taken therefore that members of the alkyl phosphate group inhibit cholinesterase by dialkylphosphorylation of the enzyme

In the past it has been assumed that both acetyl-choline and cholinesterase inhibitors combine with cholinesterase by virtue of the positively charged nitrogen, and it has been difficult to fit the alkyl phosphates into this pattern since they are predominantly negatively charged molecules and have no basic nitrogen. Adams (1949), however, has found that the essential feature of a substrate for true cholinesterase is the presence of an acetyl group, and that 3 3-dimethylbutyl acetate, which in general configuration strikingly resembles acetyl-

3 3-Dimethylbutyl acetate

choline and yet lacks the quaternary nitrogen, is split almost as fast as acetylcholine. This work strongly suggests that combination with the enzyme occurs through the negatively charged ester linkage which is, of course, present in the alkyl phosphates. It is well known that carbamyl groups are isosteric with acetyl groups and hence the activity of the carbamyl esters may be explained without recourse to the basic nitrogen. Bloch (1939) arguing in this way prepared the acetyl and isobutyryl esters of

m-hydroxyphenyltrimethylammonium (the basic half

of neostigmine) and found these esters were active

anticholinesterases

Another aspect of the problem is the stability of the inhibited complex Whereas both DFP and TEPP ultimately form apparently irreversible complexes with cholinesterase in vitro, the effects of TEPP are much shorter in duration than those of DFP in vivo If the radical transferred to the enzyme were the same, the rate of recovery from the action both in vivo and in vitro would be the same, and hence the difference in duration of action between DFP and TEPP may be due to the difference between the dissopropylphosphoryl and the diethylphosphoryl radicals If this theory is correct, the cholinesterase inhibition produced by diethylfluorophosphonate should be similar in duration to that produced by TEPP, and conversely the inhibition produced by tetraisopropylpyrophosphate should be similar in duration to that of DFP

In support of this hypothesis may be quoted the following comment from Saunders and Stacey (1948), who compared the actions of disopropyl-, diethyl-,

and dimethyl-fluorophosphonates on the eye, "We observed as early as 1941 that the dimethyl and diethyl esters produced a far less intense miosis than the dusopropyl ester The effects of the dimethyl compound wore off in a matter of hours whereas that of the diethyl compound usually lasted about 2 days The dusopropyl ester produced effects lasting usually for 7 days In the cat we (Burgen, Keele, and Slome, 1949) found that the miotic effect of TEPP lasted about 1-2 days Experiments in progress in this laboratory have shown in agreement with this theory that dissopropyl phosphoryl esters of different types have very similar durations of action to DFP, whereas other diethylphosphoryl esters have resembled TEPP in duration

The breakdown of the dialkylphosphoryl-enzyme may be simply a matter of slow uncatalysed hydrolysis and if so the isopropyl group would be expected to have a retarding influence on the reaction, alternatively the breakdown may occur through the operation of a phosphotriesterase. Aldridge (1949) has discovered a widely distributed enzyme in animal tissues which will hydrolyse diethyl p-nitrophenyl-phosphate to p-nitrophenol and diethyl phosphoric acid, and perhaps this or a similar enzyme could split off the dialkylphosphoryl group from the cholinesterase and so restore its activity

SUMMARY

- 1 The kinetics of inhibition of cholinesterase by both reversible and irreversible inhibitors is considered
- 2 Methods of estimation in common use greatly underestimate the physiological activity of the reversible cholinesterase inhibitors because of competition with substrate and the slowness with which equilibrium is attained
- 3 The action of many of the alkyl phosphate group is markedly blocked by substrate and by the presence of reversible inhibitors. Owing to this blocking by substrate the activity of this group may be overestimated in vitro.
- 4 The evidence available suggests that the alkyl phosphates form weak adsorption complexes with cholinesterase and in this activated state dialkyl phosphorylation of the cholinesterase occurs to form a stable inactive substance. This inactive

enzyme may perhaps be regenerated by hydrolysis of the dialkyl phosphoryl group the rate of which is dependent on the nature of the alkyl groups. If iso-propyl phosphoryl groups form more stable complexes with the enzyme than diethyl phosphoryl groups, this, may explain the longer duration of action of the disopropyl series in vivo

I am very grateful to Dr C A Keele and Professor F Dickens for helpful discussion I am indebted to Mr B Topley and Drs G A Mogey and F Bergel for gifts of cholinesterase inhibitors and to Dr M L Tainter for very kindly sending me a generous amount of purified cholinesterase A grant from the University of London Central Research Fund to Dr Keele enabled us to purchase the apparatus used in this work

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THE PHARMACOLOGY OF THE METHYL AND BENZYL-ESTERS OF γ-CROTONIC BETAINE (γ-CARBOXYALLYLTRIMETHYL-AMMONIUM CHLORIDE)

BY

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The effect of reversing the polarity of the ester linkage in acetylcholine homologues was first studied by Hunt and Renshaw (1926) authors prepared aliphatic esters of betaine which they showed were parasympathomimetic agents with an activity of the order of one thousandth of that of acetylcholine Betaine itself is pharmacologically almost mert and thus bears a relationship to its esters similar to that which choline bears to acetylcholine The activity of the methyl ester of β -propionic betaine, the ester most closely isosteric with acetylcholine has not so far been reported In 1928, however, a new betaine called y-crotonic betaine (y - carboxyallyltrimethyl - ammonium chloride) was isolated from animal tissues by Linneweh (1928a, b) Strack and Forsterling (1938, 1942) also isolated this betaine from mammalian muscle and prepared some of its esters found that whilst the betaine was almost inert pharmacologically, its methyl ester was a powerful parasympathomimetic drug when tested on the dorsal muscle of the leech, the frog rectus and heart and the mouse intestine They also found that the ethyl ester had much weaker effects and in addition antagonized the effects of acetylcholine The following paper confirms and extends these observations for the methyl ester and describes also the properties of the benzyl ester of this betaine Chemically the structure of the methyl ester is quite similar to that of acetylcholine, as may be seen by the formulae

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METHODS AND MATERIALS

Cats were anaesthetized with 60 mg chloralose/kg The tibialis anterior muscle was prepared for close intra-arterial injection as described by Brown (1938) The nictitating membrane response was recorded after section of the cervical sympathetic. The rabbit heart was perfused by the Langendorff method with Ringer-Locke solution. The rat phrenic nerve-diaphragm preparation (Bülbring, 1946) was set up in Tyrode solution containing 0.2 per cent (w/v) glucose and aerated with a 95 per cent O₂+5 per cent CO₂ mixture. Frog heart perfusions were carried out both by the sinus perfusion and the Straub methods. The chromodacryorrhoea response in rats was tested by the method described by Burgen (1949)

The crotonic betaine esters were prepared by the method of Bergel, Cohen, and Hindley (1949), and were made available to us as the methyl ester chloride (mol wt 1825, mp 174°C) and the benzyl ester iodide (mol wt 361, mp 147-149°C). In the text these will be referred to as the methyl and benzyl esters and the activities are given by weight. In Table I the molar activities have been used.

RESULTS

Effects on the anaesthetized cat

Methyl ester —As little as 2 μ g of the methyl ester injected intravenously produced a small fall of blood pressure, unaccompanied by bradycardia With increasing doses this effect increased, and when the dose reached 50 μ g a bradycardia accompanied the fall of blood pressure dose was increased further this effect became more marked and was associated with a decrease in the The fall of blood pressure and bradycardia were more prolonged than those given by an equivalent amount of acetylcholine (Fig 1) Sometimes the respiratory depression was preceded by a transitory respiratory stimulation This respiratory depression was unaffected by bilateral vagotomy or atropine, and respiration was shallow without the appearance of respiratory It is probable that this effect was obstruction due to a depression of the respiratory centre or the

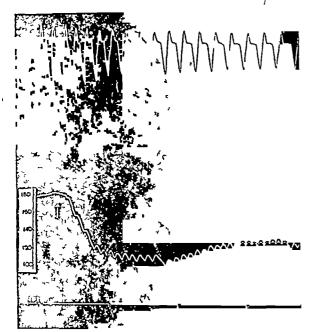


Fig 1 —Cat, 2 4 kg, chloralose Top tracing respiration Lower tracing carotid blood pressure Time in 5 sec At the arrow i v injection of 0 14 mg methyl ester

muscles of respiration Other effects noted with the larger doses of methyl ester were profuse and prolonged salivation, diarrhoea, and micturition

After an adequate dose of atropine (1–2 mg/kg) the slowing of the heart was no longer seen and the depressor effect of the methyl ester (100–200 μ g) was converted into a rise of blood pressure greater than that given by an equal dose of acetylcholine (Fig 2)

Benzyl ester —No effects were seen with the benzyl ester until 50 μ g were given A small pres-



Fig 2—Cat 24 kg chloralose Atropine, 2 mg/kg
Top tracing nictitating membrane Lower tracing
carotid blood pressure Time in 5 sec At arrow
0 2 mg methyl ester intravenously

sor effect then usually appeared which increased with increasing dosage but sometimes the response was biphasic. Respiratory depression occurred after the injection of 1 mg of the benzyl ester and was similar in character to that given by the methyl ester. At no dose level were salivation, diarrhoea or micturition observed.

In the anaesthetized rabbit similar effects were seen

Effects on the nictitating membrane

The methyl ester produced a contraction of the nictitating membrane when doses of 10 µg or more were given intravenously (Fig 2). The activity was similar to that of acetylcholine. With the benzyl ester, however, 05–1 mg was needed to produce an effect and we did not succeed in producing a maximal contraction with the maximum tolerated dose. With both esters the response was quite long lasting

Effects on the tibialis anterior preparation

Some of the effects obtained when the esters were injected intra-arterially into the tibialis anterior can be seen in Fig 3, 20 μg of the methyl ester produced a twitch similar in magnitude to that produced by 10 μg of acetylcholine but followed by a transient depression of neuromuscular conduction With 40 μg of the methyl ester the twitch was somewhat larger, relatively to nerve stimulation, but was followed by a greater neuromuscular depression. Very similar effects

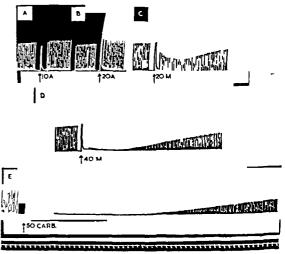


Fig 3 —Cat, 2 1 kg, chloralose Maximal motor nerve twitches of tibialis anterior muscle Close intra-arterial injection of (A) 10 μ g acetylcholine, (B) 20 μ g acetylcholine, (C) 20 μ g methyl ester, (D) 40 μ g methyl ester, (E) 50 μ g carbamylcholine, volume of injection 0 5 ml

were produced by 50 μ g carbamylcholine except that the twitch was smaller relative to the subsequent depression 250 μ g of the benzyl ester produced a twitch similar to that given by 10 μ g acetylcholine, but was followed by a prolonged neuromuscular block

Isolated preparations

Perfused rabbit heart—The addition of 1 µg of the methyl ester to the perfusion fluid exerted a negative inotropic effect about equal to that given by 0.5 µg of acetylcholine or carbamylcholine With 10 µg this effect was increased and the heart rate slowed, with 50 µg the heart was temporarily arrested and this was followed by auriculo-In these actions the ventricular dissociation methyl ester was about one quarter the activity of acetylcholine. The benzyl ester produced no depression of force or rate until 100 µg were injected, when a transitory small depression occurred After this dose, however, the response to acetylcholine was greatly decreased, and returned slowly over the succeeding 20-30 minutes

Perfused frog heart—The methyl ester depressed the force of contraction of the perfused frog heart in a concentration of about $0.01~\mu g$ /ml With ten times this dose the heart was arrested The activity relative to acetylcholme was about one tenth. The benzyl ester had no direct action on the heart even in a concentration of 1 mg/ml, but a well marked antagonism to acetylcholine was found with concentrations of the benzyl ester of $10~\mu g$ /ml and higher

Perfused vessels—Rat and guinea-pig hind limbs and the rabbit ear vessels were perfused with Locke's solution at room temperature, in all these preparations acetylcholine produced vasoconstriction. Both the methyl and the benzyl esters, given in about twice the dose of acetylcholine, produced a similar but more prolonged effect than that of acetylcholine. During the course of prolonged perfusions the sensitivity of these preparations increased equally for all three drugs.

Isolated rabbit duodenum and ileum—The methyl ester $(0.03-0.5 \, \mu g./ml)$ produced a contraction of the longitudinal muscle of the rabbit duodenum and ileum similar in magnitude to that given by one-sixth to one-half the amount of acetylcholine (Fig 4 a) The duration of the contraction was, however, longer than that given by acetylcholine, but resembled closely that produced by carbamylcholine The benzyl ester produced a contraction in the freshly isolated intestine at a concentration of 1-2 $\mu g/ml$ After the gut had

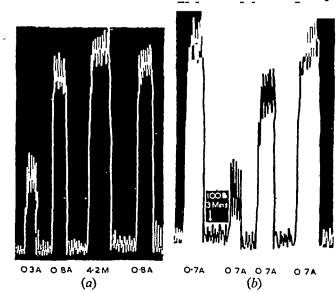


Fig 4—Rabbit duodenum. Bath volume 30 ml (a) Comparative effect of acetylcholine (0 3 μg and 0 8 μg) and methyl ester (4 2 μg) (b) Inhibition of the response to 0 7 μg acetylcholine by 100 μg benzyl ester

been isolated for one hour or more and especially if it was kept in the refrigerator overnight, the benzyl ester no longer increased the tonus, and with higher dosage a decrease in both the tonus and the spontaneous rhythm resulted. With the larger doses of the benzyl ester the motor effects of acetylcholine were antagonized, as may be seen in Fig. 4 b. When the intestine was set up as a Trendelenberg preparation, the peristalsis was completely inhibited by 10 μ g benzyl ester /ml (Fig. 5 b)

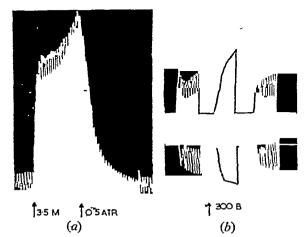


Fig 5—(a) Rabbit duodenum Bath volume 30 ml At first arrow addition of 3 5 µg methyl ester which was promptly antagonized by 0 5 µg atropine (b) Guinea-pig ileum Trendelenberg preparation At arrow addition of 0 3 mg benzyl ester for 3 minutes Upper tracing longitudinal muscle Lower tracing intestinal volume

The motor effects of both the methyl and benzyl esters were readily antagonized by atropine (Fig 5 a), and by adrenaline The benzyl ester also antagonized the motor effects of the methyl ester

Isolated guinea-pig ileum —On the guinea-pig ileum the methyl ester produced a contraction in a concentration of $0.2-1~\mu g$ /ml and was about one-half as active as acetylcholine. The benzyl ester had no direct effect on the intestine even in a concentration of 0.3~mg /ml, but at a concentration of $3~\mu g$ /ml the response to acetylcholine was reduced by 50 per cent, and with higher concentrations could be abolished. The response to histamine was unaffected until 0.3~mg /ml was added when it was depressed by about 10 per cent

Phrenic nerve-diaphragm preparation—Both the esters were fairly active neuromuscular blocking agents when tested on the phrenic nerve-diaphragm preparation. The methyl ester was about one-twentieth as active, and the benzyl ester was one-tenth as active as d-tubocurarine. Carbamylcholine was similar in potency to the methyl ester. Antagonism by neostigmine and by potassium chloride was much less marked with these esters than with d-tubocurarine. Paton and Zaimis (1949) report a similar lack of antagonism by neostigmine against the neuromuscular block produced by decamethonium iodide.

Frog rectus preparation—On the normal unsensitized frog rectus preparation, both the methyl and the benzyl esters produced a contracture similar to that produced by acetylcholine, except that the latency was longer and the rate of development of the contracture slower. When the duration of the test was the usual one and one-half minutes the methyl ester was about one-third as active, and the benzyl ester one-fifteenth as active as acetylcholine (Fig 6 a), whilst with a test lasting 10–15 minutes the relative potency of the crotonic esters was increased (Fig 6 b). Whilst the absolute sensitivity of individual rectus muscles to the crotonic esters varied very little, the sensitivity to

acetylcholine varied considerably, which accounts for the variation in relative potency

Sensitization of the muscle with eserine or TEPP increased the response to acetylcholine on an average 40 times, but produced no change in the response to either the methyl or the benzyl ester d-Tubocurarine antagonized both esters and acetylcholine about equally, but only the response to acetylcholine was restored by eserine

Rat chromodacryorrhoea

Albino rats (c 300 g wt) were used which gave no tear secretion after the injection of 250 μg acetylcholine subcutaneously, but a well-marked red tear secretion to 500 μg acetylcholine. The following results were obtained with the methyl ester

20 μg—no response

50 μg—pink tinging of the tears after 5-6 minutes

100 μg —profuse opaque red tears—some bradycardia

1,000 µg —profuse and very prolonged red tear secretion, heart block, profuse salivation, respiratory difficulties, spontaneous micturition and diarrhoea

Doses of up to 20 mg of the benzyl ester were ineffective in causing a red tear secretion

In Table I the activities of the methyl and benzyl esters are summarized and compared on a molar basis with those of acetylcholine

Hydrolysis by cholinesterases

The esters were incubated in 10-5 concentration at 37° C either with a solution of lysed human red cells as a source of true cholinesterase or with human serum as a source of mixed esterases. The residual ester was estimated on the frog rectus preparation

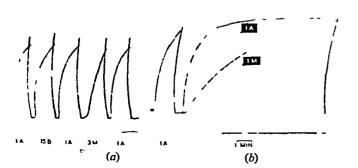


Fig 6—Frog rectus abdominis (a) Comparison of 1 μg acetylcholine with 15 μg benzyl ester and 3 μg methyl ester Duration of test 90 sec (b) Comparison of 1 μg acetylcholine and 1 μg methyl ester Duration of test 10 min

TABLE I COMPARATIVE ACTIVITY OF THE METHYL AND BENZYL ESTERS

Preparation	Molar activity relative to acetylcholine = 100			
	Methyl ester	Benzyl ester		
Frog-rectus unsensitized ,, ,, sensitized with	30–100	15-50		
eserine	2–5	0 5-2		
Guinea pig ileum	50	0*		
Rabbit duodenum†	15–50	5**		
Perfused rabbit heart‡	10–50	0*		
Frog heart	10	0*		
Cat blood pressure	8	1§		
,, ,, ,, after atropine	250	20		
Cat nictitating membrane	80	0.5_		
Cat tibialis ant (twitch)	50	8		
Rat diaphragm	1	511		
Perfused vessels	50"	20-100		
Rat red tear secretion	1,000	0		
•	1			

^{*} Blocks action of acetylcholine ** Blocks action of acetylcholine and decreases intestinal tone (see text) § Biphasic or pressor response decreases intestinal tone (see text) § Biphasic or pressor response decreases intestinal tone (see text) § Biphasic or pressor response decreases intestinal tone carbamylcholine, 150 † Activity of carbamylcholine 100

From the results given in Table II it will be seen that a preparation of true cholinesterase which hydrolysed acetylcholine very rapidly did not hydrolyse either ester. Serum did increase the breakdown of the benzyl ester, but at a rate far lower than for acetylcholine. The non-enzymatic

TABLE II

HYDROLYSIS OF METHYL AND BENZYL ESTERS BY

CHOLINESTERASES

		% Hydrolysed					
Ester	Enzyme	10 min	1 hr	2 hrs	3 hrs	6 hrs	
Benzyl ester	No enzyme 5% serum* 1% R B C *		5 5 -1	 	12 18 2	24 28 1	
Methyl ester	No enzyme 5% serum* 1% R B C *	0 0 3		2 0 3		-	
Acetyl- choline	5% serum 1% R B C	97 99	_	100 100		_	

^{*} Corrected for non-enzymatic hydrolysis

rates of hydrolysis of the benzyl ester and of acetylcholine were measured by continuous titration over the range pH 45-95. The velocity constants were similar at the lower pH values, but activation by hydroxyl ions occurred sharply at pH 75 with the benzyl ester and at a slightly higher pH and less steeply with acetylcholine.

In experiments carried out in the Warburg apparatus it was found that $0.002 \, M$ methyl ester-inhibited red cell cholinesterase 23 per cent (substrate $0.025 \, M$ acetyl- β -methylcholine chloride) and inhibited serum pseudo cholinesterase 18 per cent (substrate $0.007 \, M$ benzoylcholine chloride) These data show that both true and pseudo cholinesterases have an affinity for the methyl ester of the same order as that for acetyl- β -methyl choline and benzolycholine. It would seem that reversal of the ester link, whilst not reducing the affinity of cholinesterase for the methyl ester, prevents the enzyme-substrate complex breaking down into γ -crotonic betaine and methanol

SUMMARY

The *methyl* ester of γ -crotonic betaine is a very active parasympathomimetic drug. Both muscarinic and nicotinic types of action were found whose activity varied from one fiftieth to ten times that of acetylcholine according to the test preparation. The ester is not split by cholinesterase and hence has a more prolonged action than acetylcholine. The *benzyl* ester of γ -crotonic betaine has almost no muscarinic actions, but has some weak atropine like activity. Compared with the the methyl ester the nicotine actions are considerably weaker

We are grateful to Dr F Bergel for placing a generous supply of these esters at our disposal

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THE METHYLATION OF NORADRENALINE BY MINCED SUPRARENAL TISSUE

BY

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It has now been established that the pressor activity in extracts from the suprarenal glands is not due to the presence of adrenaline only but also to the presence of noradrenaline. This has been shown for the pig's gland by Schumann (1948, 1949) and for the dog's gland by Bülbring and Burn (1949a) by biological estimation That both substances are present in extracts of suprarenal glands from cattle was shown by v Euler and Hamberg (1949) who compared the results obtained by biological methods with those obtained by paper chromatography (James, 1948) which they modified to yield quantitative results Goldenberg, Faber, Aston, and Chargaff (1949) have also applied chemical and biological methods to show the presence of noradrenaline in commercial extracts of adrenal medulla from cattle and found that these contained 12-18 per cent noradrenaline, one sample contained as much as 36 per cent. Tullar (1949) has isolated l-noradrenaline from such extracts

The presence in the gland of the non-methylated compound may have two purposes. It may be an end-product which can be released into the blood-stream, it may also be the precursor for the synthesis of adrenaline by the gland.

There is recent evidence for the release of noradrenaline itself from the suprarenal medulla as was first suggested by Meier and Bein (1948) According to Holtz and Schümann (1949) the suprarenal gland releases a substance which is not adrenaline and is most probably noradrenaline during the pressor reflex following clamping of the carotid arteries Bülbring and Burn (1949b) showed that in the eviscerated cat stimulation of the splanchnic nerve to the suprarenal caused release of a mixture of adrenaline and noradrenaline, this was shown by the fact that the pressor effect of splanchnic stimulation could be matched by an infusion of adrenaline, but the ratio of contractions of the two nictitating membranes, one denervated and one normal, could only be matched by infusing a mixture of adrenaline and noradrenaline In order to see whether the admixture of *nor*adrenaline was due to deficient methylation a number of cats were fed with methionine. However, in these cats also splanchine stimulation produced a release of *nor*adrenaline with the adrenaline. The only difference observed was that the gradual decline in the proportion of adrenaline secreted during repeated splanchine stimulation seemed to be absent in the animals fed on methionine.

This result suggested that the suprarenal gland, though normally releasing noradrenaline to exert a function of its own, was also capable of methylating noradrenaline in order to increase its store of adrenaline. The likelihood of this mechanism had been put forward by Blaschko (1939, 1942)

The present paper is concerned with the conversion of *nor*adrenaline to adrenaline by minced suprarenal glands *in vitro*

Метнор

Dogs and cats were used The suprarenal glands were obtained by three different procedures The first was to anaesthetize the dog quickly with ether, bleed it and remove the gland with the shortest possible delay The second was to anaesthetize the dog with ether, open the abdomen and remove the glands by operation while the dog was kept alive The third was used in cats Under ether anaesthesia the cord was cut, the brain destroyed and artificial respiration was given In some experiments the right splanchnic nerve was dissected by retroperitoneal approach and was cut Then the left splanchnic nerve was similarly dissected and stimulated In other experiments both splanchnic nerves were stimulated simultaneously After 30 minutes' stimulation the cat was bled out and the glands were removed with the shortest possible delay

The suprarenals were dried with filter paper and weighed. They were then transferred into an icecold mortar, cut with scissors and ground with icecold saline, 1 c c per 0.05-0.1 g gland. Of this suspension 2 c c were transferred to a test tube, 0.3 c c M/15 phosphate buffer was added, and in different experiments varying amounts of choline chloride, racemic noradrenaline hydrochloride and adenosine triphosphate (ATP) were

added Each tube thus contained

1

20 c c extract containing 01-02 g gland

03 cc M/15 phosphate buffer

0 3-0 6 c c containing varying amounts of choline chloride

, 03-06 cc containing varying amounts of noradrenaline

04cc containing 8 mg ATP

33-39 cc total volume

The weight of choline chloride added was always the same as that of racemic noradrenaline hydrochloride, 1e, twice the amount of l-noradrenaline experiments in which no noradrenaline was added the amount of choline added will be specifically stated If any of the ingredients was omitted the corresponding volume of saline was added instead For each experiment one or several control samples were prepared the final procedure adopted was to add 0 3 c c buffer and 1 c c N HCl to 2 c c extract, this was quickly heated to boiling and immediately cooled The same ingredients were added to this boiled control sample as to the one with which it was to be compared, the two were incubated alongside The usual incubation time was 1 hour After incubation 1 c c N HCl was added to each sample. though not to the control sample if acid had been added before incubation The sample was then transferred to an Erlenmeyer flask One washing with 2 c c buffer and two washings with 2 c c saline were also transferred into the Erlenmeyer flask Each acidified sample was quickly heated to boiling point, cooled and made up to such a volume that 01 g gland was present in 10 c c This solution was used for the assays

In some experiments samples were incubated in Thunberg tubes evacuated and filled with nitrogen. The assay was carried out by the following methods

The rat uterus method (de Jalon, Bayo, and de Jalon, 1945) This assay is based on the antagonism between adrenaline and acetylcholine Constant contractions are evoked by constant doses of acetylcholine at constant intervals. The activity of the samples is estimated by the reduction in the size of contraction compared with that produced by a standard adrenaline solution preparation is almost invariably 100 times less sensitive to noradrenaline than to adrenaline It has been discussed in detail in a recent paper by Gaddum, Peart, and Vogt (1949) and I have used the same procedure I am indebted to Professor Gaddum for the suggestion of using an apparatus according to Schild (1947) with a solution of carbachol instead of acetylcholine In the later part of the experiments a simplified automatic apparatus has been used I am indebted to Mr O B Saxby for its construction

The frog heart, in which the ratio N/A of activity of noradrenaline to adrenaline is not as low as in the rat uterus, was used in one experiment only

The rabbit's duodenum was used in 3 experiments. The ratio N/A for the rabbit's duodenum varied between 1 and 2. Adrenaline was never found to be the stronger

The spinal cat's blood pressure was used in all except two experiments. The ratio N/A was very variable. In 3 out of 15 experiments it was less than 1 and only in 2 cats was it 2. The range was from 0 615 to 2.

The cat's nictitating membrane This method was used in 11 out of 15 experiments. It has been described in detail in a recent paper by Bülbring and Burn (1949b) and is based on the fact that the denervated nictitating membrane becomes relatively much more sensitive to noradrenaline than the normal membrane which is more Thus the ratio of the size of sensitive to adrenaline contraction by the denervated membrane to that by the normal membrane is greater the larger the proportion of noradrenaline in the mixture By this means it is possible to test the activity of an extract in a spinal cat not only by the effect on the blood pressure, comparing it with adrenaline, but also on the nictitating membranes by comparing it with an equipressor dose of a known mixture of adrenaline and noradrenaline

For the calculation of the results obtained by different methods of different sensitivities a formula has already been used by Professor J H Gaddum (personal communication) I am grateful to Mr J St L Philpot for his advice in developing a formula as follows. Adrenaline was used as a standard throughout. Thus in a preparation of high sensitivity to adrenaline and of low sensitivity to noradrenaline (e.g., rat uterus and frog heart) let 1 μ g noradrenaline be equivalent to "a" μ g adrenaline. In a preparation of similar sensitivity to both let 1 μ g noradrenaline be equivalent to "b" μ g adrenaline. Let the total activity on the rat uterus or frog heart be "U" (expressed as μ g adrenaline) and let the total activity on the rabbit's intestine or cat's blood pressure be "C" (expressed as μ g adrenaline). Then

$$U = A + aN$$
$$C = A + bN$$

where A is μg adrenaline and N is μg noradrenaline From these two equations the following formulae are derived

$$A = \frac{bU - aC}{b - a}$$

$$N = \frac{C - U}{b - a}$$

These formulae were used for the calculations, of which two examples will be given

The amount of *nor* adrenaline will be given throughout in terms of *l-nor* adrenaline

Example 1

The estimation by the rat uterus method gave the following results for "U" in μ g adrenaline per c c

	Sample I	Sample II
1st strip	2 55	63
2nd strip	2 27	4 0
Mean "U"	2 41	5 15

The estimation on the cat's blood pressure gave for Sample I "C" = 27 2 μ g adrenaline per c c Sample II "C" = 25 8 μ g adrenaline per c c

The ration of activity $\frac{noradrenaline}{adrenaline}$ was on the rat's uterus "a" = 0 01, on the cat's blood pressure "b" = 18 From these results the values for adrenaline and "noradrenaline were calculated



Fig 1—Assay of suprarenal extracts (Exp 1) on spinal cat Tracings are arterial blood pressure (bottom), contractions of denervated (middle) and normal (top) nictitating membrane. Figures above are ratios of the size of contractions of the two membranes. I and II refer to samples in Exp 1 "A" followed by a number indicates the percentage of adrenaline in a mixture of adrenaline and noradrenaline.

$$A_{1} = \frac{18 \times 241 - 001 \times 272}{18 - 001}$$

$$= \frac{435 - 027}{179} = 227 \,\mu\text{g} \text{ adrenaline}$$

$$N_{1} = \frac{27.2 - 241}{18 - 001} = 139 \,\mu\text{g} \text{ noradrenaline}$$

$$A_{11} = \frac{18 \times 515 - 001 \times 258}{18 - 001}$$

$$= \frac{930 - 025}{179} = 508 \,\mu\text{g} \text{ adrenaline}$$

$$N_{11} = \frac{258 - 515}{18 - 001} = 115 \,\mu\text{g} \text{ noradrenaline}$$

Another estimate was obtained by recording the contractions of the cat's nictuating membranes and comparing the extracts with mixtures containing varying proportions of adrenaline and noradrenaline. On top of the tracing shown in Fig. 1 are given the ratios of the size of contractions of the denervated to those of the

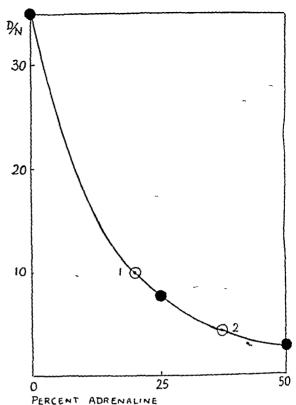


FIG 2—Curve relating ratio of the size of contractions of the denervated to the normal nicitating membrane (ordinates) and the percentage adrenaline in mixtures with noradrenaline. The black circles represent effects obtained with standard solutions, white circles those obtained with suprarenal extracts

normal membrane The ratio for Sample II was between those for two mixtures containing 25 per cent and 50 per cent adrenaline respectively The ratio for Sample I was less than that for the mixture containing 25 per cent adrenaline The actual percentages in the extracts were obtained graphically from a curve relating the ratio of contractions of the nictitating membranes to the percentage adrenaline present in the standard solution injected (see Bülbring and Burn, 1949b) When this was done, as shown in Fig. 2, the percentage for those injections of extract, shown in Fig 1, were 20 per cent adrenaline in Sample I and 37 per cent adrenalme in Sample II Though the pressor effects in this stage of the experiment were gradually diminishing it may be seen that 0.5 c.c sample II was $> 8 \mu g$ mixture containing 25 per cent adrenaline and $< 9 \mu g$ mixture containing 50 per cent adrenaline

1 c c sample II \equiv 17 μg mixture containing > 25 and <50 per cent adrenaline

0 45 c c sample I \equiv 8 μg mixture containing 25 per cent adrenaline

1 c c sample I \equiv 17 8 μ g mixture Estimations of this kind were repeated several times and the mean results were that Sample I contained 21 per cent adrenaline in 17 76 μ g total activity

Sample II contained 34 per cent adrenaline in 17 5 μ g total activity

As the samples represented a 1 in 100 dilution of the suprarenal tissue the activity could be calculated as μg per g gland. This is shown in Table I

TABLE I
ACTIVITY IN SUPRARENAL EXTRACTS

		• Preparation	μg per g gland			
Sample	Sample Esu-	used for estimation	Adren- aline	Nor- adren- aline	Total	
I	1st 2nd	Rat uterus and cat blood pressure Cat nictitating membrane and	227	1,390	1,617	
		blood pressure Mean	372 300	1,404 1,397	1,776 1,697	
	1st	Rat uterus and cat blood pressure	508	1,150	1,670	
п	2nd	Cat nictitating membrane and blood pressure Mean	596 552	1,154 1,152	1,750 1,704	

The agreement of the results obtained by the different methods was very close. It was so in 10 out of 15 experiments. Estimations of total activity did not differ from the mean figure of several combined assays by more than 10 per cent in 14 out of 15 experiments. The deviation from the mean figure for the adrenaline content was more than 20 per cent in 4 out of 15 experiments. One of these will be taken as the second example.

Example 2 (This was the experiment in which the difference between the two estimates was greatest.)

The assay by the rat uterus method gave widely differing results for "U" in μg adrenaline per c c

	Sample	I Sample II	Sample III
1st strip	2 25	48	2 05
2nd strip	29	4 85	24
3rd strip	5 65	98	4.25
Mean	36	65	29

The assay on the cat's blood pressure gave the following results for " C"

1 c c Sample I equivalent to 48 μg adrenaline

1 cc Sample II ,, ,, $34 \mu g$, 1 cc Sample III ,, ,, $44 \mu g$,

With "a" = 0 01 and "b" = 2 0 the activity in μ g per g gland was calculated and is shown as the first estimate for each sample in Table II The much weaker pressor activity of Sample II than that of Sample I, when compared with adrenaline, is shown in Fig 3

TABLE II
ACTIVITY IN SUPRARENAL EXTRACTS

	Preparation		μg per g gland			
Sample	Sample mate used for estimation	Adren- aline	Nor- adren- aline	Total		
I	1st 2nd	Rat uterus and cat blood pressure Cat nictitating membrane and	340	2,230	2,570	
		blood pressure Mean	840 590	1,560 1,895	2,400 2,485	
п	1st 2nd	Rat uterus and cat blood pressure Cat nictitating	640	1,400	2,040	
		membrane and blood pressure Mean	1,170 905	1,130 1,265	2,300 2,170	
m	1st 2nd	Rat uterus and cat blood pressure Cat nictitating	270	2,080	2,350	
111		membrane and blood pressure Mean	550 410	1,650 1,865	2,200 2,275	

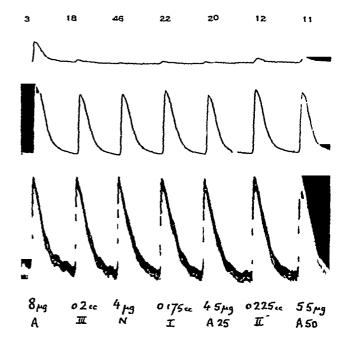


Fig 3—Assay of suprarenal extracts (Samples I, II, and III in Exp 2) on spinal cat Records as in Fig 1

 $8 \mu g$ adrenaline = $4 \mu g$ noradrenaline

- = 4 5 μg mixture containing 25 per cent adr
- = 5 5 μ g mixture containing 50 per cent adr
- = 0 175 c c Sample I
- = 0.225 cc Sample II
- = 02 c c Sample III

With these small doses, which were suitable for the assay of pressor activity, the normal nictitating membrane was scarcely affected. Larger doses had to be given for this purpose and part of the assay is illustrated in Fig. 4. By graphical determination from curves similar to that shown in Fig. 2 the proportion of adrenaline in the samples according to Figs. 3 and 4 were as follows

	Sample I	Sample II	Sample III
Fig 3	22 per cent	48 per cent	29 per cent
Fig 4	38 ,, ,,	50 ,, ,,	16 ,, ,,

The mean figures of all estimations in this experiment are shown as the second estimates in Table II The discrepancy between these and the other parallel assay is more than 100 per cent. However, the change in the three samples is in the same direction and of the same magnitude. This was so in every experiment without exception. No matter how large the absolute difference was between various assays for one sample (and it was never as large as in this sample), the relative difference between the samples was not affected, they always differed in the same direction. As there was no reason for assuming that either the assay combining results obtained on rat uterus and cat's blood pressure, or the assay in which extracts were compared with known mixtures on the

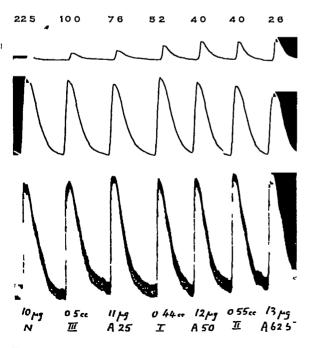


Fig 4 —Assay (Exp 2) continued Records as in Fig 1.

nictitating membranes was more accurate than the other, the mean figure of both estimates was taken. The results were all converted to μg per g gland and are thus represented in the Tables

RESULTS

1 The establishment of the control figure

In order to observe a change in the proportion of adrenaline and noradrenaline in a sample of suprarenal extract a control sample must be available which is subjected to the same conditions but in which enzyme activity has stopped A comparison was therefore made on several occasions between extracts (a) acidified, boiled, incubated and not incubated, (b) non-acidified, boiled and then incubated, (c) the same, but with various additions The results are given in Table III which shows the close agreement obtained by the combination of several biological methods The total amount found in boiled control samples after one hour incubation did not differ by more than 6 per cent. from the samples which were not incubated agreement was close irrespective of whether they were acidified or not A larger difference was found in Exp 2 in the calculated total when a sample to which noradrenaline had been added was compared with another without addition the estimations of the amount of adrenaline and noradrenaline in several samples of the same gland differed up to 20 per cent, the proportion of the two remained the same within 7 per cent The largest differences were again found between estimations of samples to which noradrenaline had been added and those without (Exps 2 and 6), and also between samples incubated in O₂ and N₂ (Exps 10 and 11) For this reason the control samples had the same additions and were subjected to the same conditions as those with which they were compared The only exceptions are in experiments 4 and 5

2 The conversion of noradrenaline to adrenaline

Eight experiments were performed on dogs' suprarenals, the results of which are summarized in Table IV The first three glands were removed by operation under ether anaesthesia, the other glands were removed as quickly as possible while the dog was bled out In Exp 6, in which the dog was used as blood donor for a heart-lung preparation, the delay before removal of the glands was considerable, this may be the reason for no change taking place during incubation. In all the other experiments a conversion of noradrenaline to adrenaline was observed. This can only be assumed if together with the increase in adrenaline a corresponding decrease in noradrenaline is found. Thus the values for

TABLE III

DOGS' SUPRARENALS COMPARISON OF CONTROL SAMPLES

Amounts of adrenaline (adr) and noradrenaline (nor) are given in μ g per g gland

No of expen-	Acıdı boıled addıtı <i>Not</i> ıncı	l, no ons	Not a fied, b ATP a Incub	oiled, idded oated	Not acidified, boiled, ATP and choline added Incubated in O ₂		Not acidified, boiled, ATP, choline and noradrenaline added Incubated in O ₂		Acidified, boiled, A'choline and noradrer added Incubate		aline d	Deviation of estimations from mean	
	μg	%	μg	%	μg	%	μg	%	μg	%	μg	%	%
Adr (2) <i>Nor</i> Total			645 810 1,455	44 56	640 905 1,545	41 59	655 690 * 1,345	48 52			•		$\begin{bmatrix} -1 & 2 & +1 & 3 \\ -13 & +13 & \\ -7 & 5 & +7 & 2 \end{bmatrix}$
Adr (3) <i>Nor</i> Total	550 1,875 2,425	23 77			580 2,000 2,580	22 5 77 5							±27 ±32 ±31
Adr (6) <i>Nor</i> Total	635 365 1,000	63 5 36 5			643 353 996	65 35	680 320* 1,000	68 32					-28, +42 -75, +55 ±02
Adr (10) Nor Total					,		1,073 1,210 2,283	47 53	900 1,270 2,170	42 58	1,120 1,175 2,295	49 51	-15, +5 $-35, +43$ $-35, +21$
Adr (11) Nor Total							1,050 1,770 2,820	37 63			1,200 1,700 2,900	41 59	±73 ±20 ±14

^{*} The amount of noradrenaline added has been subtracted in order to make the sample comparable with the other samples of the same gland

"Percentage change in adrenaline content" are calculated by taking into account not only the amount of adrenaline estimated but also the amount of noradrenaline In Exp 1 an increase of 588 μ g adrenaline was found, but the decrease of noradrenaline was only 506 μ g The conversion is thus not more than 506 μ g or an increase of 99 per cent on the original amount of 511 μ g adrenaline

The presence of ATP was found to be essential for the methylation of noradrenaline. In four experiments samples incubated with ATP showed an increase of adrenaline, whereas parallel samples without ATP showed a decrease, which in Exp 10 was accompanied by a corresponding increase in noradrenaline, indicating the possibility of demethylation. The addition in one experiment (No 2) of ATP alone caused no change in the proportion of adrenaline to noradrenaline.

The amount of *nor*adrenaline present in the samples had an important influence though it did not wholly govern the extent of the reaction Exp 2 offers a good example Sample a, to which only ATP was added, showed no change Sample b, to which both ATP and choline were added, showed an increase in adrenaline which was similar

to that of sample c to which noradrenaline was added as well None of the added *nor*adrenaline seemed to have been used In two experiments (2b) and 3a) in which the original gland was rich in noradrenaline, a conversion to adrenaline was observed though only ATP and choline were added But in two other experiments in which the gland contained originally very little noradrenaline (5a) or none (4a) no increase in adrenaline was observed In the latter experiment there is a further indication for a conversion in the opposite direction noradrenaline was added to the samples as well (4b) and 5c) some increase in adrenaline content was observed in both experiments, though surprisingly Little considering the large amount added (2,000 μ g per g gland) In Exp 5b a sample incubated with ATP and noradrenaline, but omitting the choline, nevertheless methylated a similar amount to that methylated in the one to which choline had been added

There were thus four points emerging from Table IV (1) ATP was essential for the conversion, (2) conversion was possible if ATP and choline were added under certain conditions, (3) these conditions depended to some extent on the amount of nor-

TABLE IV

DOGS' SUPRARENALS

Amounts of adrenaline and noradrenaline before and after incubation in μg per g gland

No of	Original			Final			Change			Percentage change in	Additions before incubation		
Exp	Adr	Nor	Total	Adr	Nor	Total	Adr	Nor	Total	adr content	ATP	Ch	Nor
1	511	912	1,423	1,099	406	1,505	+588	-506	+82	+99	+	+	833
2 <i>a</i> † <i>b c</i>	645 640 655	810 905 1,940	1,455 1,545 2,595	615 1,110 1,100	745 350 1,570	1,360 1,460 2,670	-30 +470 +445	-65 -555 -370	-95 -85 +75	-5 +73 +57	+++	++	_ 1,250
3a‡ b‡	} 580	2,000	2,580	{ 750 450	1,750 1,970	2,500 2,420	+170 -130	-250 -30	-80 -160	+29 -17	+ -	+	
4a‡	1,340 1,340	0 2,000*	1,340 3,340	1,130 1,515	225 1,695	1,355 3,210	-210 +175	+225 -305	+15 -130	-15 +13	++	+	2,000
5a‡ b c d	1,048	212 2,212*	1,260 3,260	1,040 { 1,308 1,358 845	224 1,820 1,816 2,120	1,264 3,128 3,174 2,965	$ \begin{array}{r} -8 \\ +260 \\ +310 \\ -203 \end{array} $	+12 -392 -396 -92	+4 -132 -86 -295	0 +25 +30 -10	+++	+ - + +	2,000 2,000 2,000
6	680	1,320	2,000	695	1,385	2,080	+15	+65	+80	+2	+	+	1,000
10 <i>a b</i>	983	1,238	2,221	∫ 1,170	1,100 1,465	2 270 2,218	+187 -230	-138 + 227	+49 -3	+15 -23	+	+ +	500 500
11 <i>a b</i>	} 1,050	1,770	2,820	{ 1,290 1,047	1,385 1,664	2,675 2,711	+240 -3	-385 -106	-145 -109	+23	+ -	++	1,000 1,000

^{*} Values are calculated not estimated. † Choline chloride addition was 2.5 mg per g gland ‡ Choline chloride addition was 4 0 mg. per g gland

TABLE V CATS' SUPRARENALS $s = splanchnic nerve stimulated, o = no stimulation, adr, nor, and total in <math>\mu g$ per g gland

No of O		Original		, Final -			Change			Percentage change in	Additions before incubation		
Exp	Adr	Nor	Total	Adr	Nor	Total	Adr	Nor	Total	adrenaline content	ATP	Çh	Nor
7 <i>a</i> s <i>b</i> o	360 452	3,685 3,353	4,045 3,805	480 630	3,408 3,225	3,888 3,855	+120 +178	-277 -118	-157 +50	+33 +26	++	++	3,000 3,000
8 <i>a</i> †s <i>b</i> †o	485 617	870 363	1,355 980	765 598	450 372	1,215 970	+280 -19	-420 +9	-140 -10	+58 -2	++	++	- -
9*as b o		1,173 1,745	1,550 2,889	447 1,164	1,271 1,817	1,718 2,981	+70 +20	+98 +72	+168 + 92	0	++	++	540 620
12a s b s	1,000	3,260	4,260	{ 1,790 820	2,500 3,400	4,290 4,220	+790 -180	-760 +140	+30 -40	+76 -14	+	+++	1,500 1,500
13a s b s	1 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2	1,650	2,072		1,465 - 1,545	2,080 1,967	+193 0	-185 -105	+8 -105	+44	+	+ +	1,500 1,500
14a s b s		1,397	1,697		1,152 1,242	1,704 1,529	+252 -13	-245 -155	+7 -168	+82	+	+++	1,500 1,500
15a s b s		1,895	2,485		1,265 1,865	2,170 2,275	+315 -180	-630 -30	-315 -210	+53	+	++	1,500 1,500

^{*} The samples in this experiment were incubated in N₂

[†] Choline chloride addition was 1 mg. per g. gland

adrenaline present but the reaction was probably limited by the amount of enzyme available, (4) the addition of the three ingredients—ATP, choline, and noradrenaline—caused a conversion in 6 out of 7 samples

A surprising result was that glands removed under the most favourable conditions converted less noradrenaline into adrenaline than those removed during a prolonged operation. As it is known that there is an increased splanchnic discharge during ether anaesthesia and laparatomy, the effect of splanchnic stimulation was investigated

Cats were used for this purpose and the results are summarized in Table V In the first three experiments the splanchnic nerve to one gland had been stimulated but not to the other, both glands were incubated under the same conditions with the addition of ATP, choline, and varying amounts of Of the latter an excessively large noradrenaline amount was added in Exp 7 and a conversion was observed in both glands, which was slightly more on the stimulated side In Exp 8 no noradrenaline was added before incubation and a large difference was seen between the activity of the stimulated as compared with the non-stimulated side Though the total activity of the stimulated gland was not less than that of the non-stimulated side (in fact it was 37 per cent higher) the proportion of adrenaline When both suprarenal extracts was much less were incubated with ATP and choline the proportion on the stimulated side became the same as that of the other side in which it remained unchanged This is shown in Table VI In Exp 9 (Table V) no

TABLE VI SUPRARENAL EXTRÀCTS INCUBATED WITH ATP AND CHOLINE

, Gland	Original Adr <i>nor</i> adr	-Final Adr <i>nor</i> adr
Stimulated	36 64	63 37
Non-stimulated	63 37	62 38

change was observed on either side, though the amount of adrenaline was increased there was no corresponding decrease of noradrenaline. The result is not clear because the total activity was found to be considerably more than that of the control samples. This was the only experiment in which the control samples were heated in a water-bath for 5 min instead of bringing them to boiling point quickly. Also it was the only experiment, recorded in Table V, in which the samples were incubated in nitrogen. This point will be discussed later. On the other hand, it may have been that too small an

amount of noradrenaline was added for this gland

In the last four experiments in Table V both splanchnic nerves were stimulated, both suprarenals were pooled and the extracts incubated with an amount of noradrenaline which was expected to be well above the amount of adrenaline present in the gland after prolonged splanchnic stimulation. This expectation was fulfilled as in each experiment the amount of noradrenaline was found to be 3-4 times that of adrenaline. In each experiment the increase in the amount of adrenaline after incubation was considerable, i.e., 76, 44, 82, and 53 per cent. However, in parallel samples to which no ATP had been added no such increase was observed. One of these samples (in Exp. 12) gave yet another indication of a conversion in the opposite direction.

The experiments on cats' suprarenals confirmed the observation that the presence of ATP is essential for the methylation of *nor*adrenaline. The glands in which the store of adrenaline had been depleted by previous splanchnic stimulation appeared to have a high enzyme activity

It might be argued that any change in adrenaline should not be calculated as a percentage change of adrenaline initially present in the gland but rather This would, however, give of the *nor*adrenaline misleading results as the initial amount of noradrenaline depended largely on the very variable amounts added to different samples For example, ın sample 2b (Table IV) 470 μg adrenalıne was formed which is a conversion of 52 per cent of the initial 905 μ g noradrenaline, while in sample 2c (of the same gland) the very similar formation of 445 µg adrenaline represents only 23 per cent of the ınıtıal 1940 µg noradrenaline which was as high as this because 1,250 μ g had been added Secondly, though splanchnic stimulation depleted the store of adrenaline the absolute figures of the initial content are on the whole similar to those of unstimulated glands and the highest absolute increase of 760 μ g occurred in a stimulated gland containing initially as much as $1,000 \mu g$ adrenaline per g (Exp 12a, Table V)

Assuming that through the prolonged operative procedure in the first three dog experiments there occurred a certain amount of splanchnic stimulation the results may be summarized as in Table VII (Exps 6 and 9 have been omitted from this table as the absence of any change in either direction could probably be accounted for by faulty experimental procedure discussed above) In glands depleted by splanchnic stimulation the increase in adrenaline content was larger than the variation found in control samples (see Table III) In 7 out of 10 samples it was more than 50 per cent and the mean

TABLE VII
EFFECT OF SPLANCHNIC STIMULATION

Stimulated glands			Non-stimulated glands		
No of exp	Percentage change in adrenaline total content activity		No of exp	Percentage c adrenaline content	hange in total activity
DOGS 1 2b 2c 3a	+99 +73 +57 +29	+ 58 - 55 + 29 - 31	4b 5b 5c	+13 +25 +30	-3 1 -3 2 -2 6
			10a 11a	+15 +23	+22 -51
CATS 7a 8a	+33 +58	- 3 9 -10 4	7 <i>b</i> 8 <i>b</i>	+26 - 2	-1 3 -1 2
12 <i>a</i> 13 <i>a</i> 14 <i>a</i> 15 <i>a</i>	+76 +44 +82 +53	+ 07 + 04 + 08 -126			
Mean	+60 5		Mean	+18 6	

figure for all was an increase of 60 5 per cent from the original On the other hand, in non-stimulated glands, no increase above 30 per cent was observed, only in 4 out of 8 experiments was it larger than the variation determined in control samples, and the mean figure for all was an increase of only 18 6 per cent The variation in total activity was on an average no more than between several control

TABLE VIII EFFECT OF ATP

	Suprarenal extracts incubated					
No of exp	With Percentage adrenaline content		Withou Percentage adrenaline content			
DOGS 3 5 10 11	+29 +30 +15 +23	- 3 1 - 2 6 + 2 2 - 5 1	-17 -10 -23 0	- 62 - 91 - 01 - 39		
CATS 12 13 14 15	+76 +44 +82 +53	+ 07 + 04 + 08 -126	-14 0 0 0	- 09 - 51 -100 - 85		
Mean Dogs Cats	+24.25 +63.75		-12 5 - 3 5	-		

samples However, in two samples (Table VII, Exps 8a and 15a) there was a loss of 104 and 126 per cent respectively Nevertheless, in spite of this loss in total activity, the proportion of adrenaline was increased by 58 and 53 per cent respectively

The experiments in which samples of the same gland were incubated with and without ATP are summarized in Table VIII While there was always an increase of adrenaline when ATP was present there was either no change or a loss when ATP was absent In Exp 10, where the increase in adrenaline might not be significant it becomes more so when the loss of 23 per cent in the parallel sample containing no ATP is considered. On the average the loss in total activity does not exceed the variation in several control samples shown in Table III, but the samples containing no ATP were without exception found to be weaker than their control samples The loss never exceeded 10 per cent

3 Experiments in aerobic and anaerobic conditions

In four experiments a comparison was made between samples incubated in air and incubated in nitrogen. The results are shown in Table IX. It appears that methylation takes place anaerobically but not to the same extent as aerobically. Though the increase in the first two experiments is small and

TABLE IX

EFFECT OF AEROBIC OR ANAEROBIC INCUBATION

No	Percentage change in adrenaline content in suprarenal extracts incubated					
of exp	In air With ATP Without ATP		In nitrogen With ATP Without ATP			
10 11 13 14	+15 +23 +44 +82	-23 0 0 0	+2 +17 +24 +58	-22 3		

may not be significant, there is both in aerobic and anaerobic conditions a clear difference between samples incubated with and without ATP. The conclusion is drawn that anaerobic conditions are less favourable for the methylation of noradrenaline and that this may be partly responsible for the negative result in Exp. 9 (Table V)

4 The loss of total activity during prolonged incubation

One experiment was carried out in which samples were incubated for different times. The loss was considerable, as shown in Table X. It may be seen

TABLE X
EFFECT OF DURATION OF INCUBATION
Dog's suprarenal, µg per g gland Change during incubation

, C	Control			For 1 ho	our		For 1½ ho	ours	-	For 2½ ho	ours
	%	μg	%	μg	Change	%	μg	Change	%	μg	Change
Adr	22 5	580	30	750	+170	30	730	+150	33	690	+110
Nor Total	_77 5	2,000 2,580	70	1,750 2,500	-250 -80	70	1,690 2,420	-310 -160	67	1,410 2,100	-590 -480

that nearly 20 per cent of the total activity was lost in $2\frac{1}{2}$ hours. Though the proportion of adrenaline to noradrenaline remained approximately the same up to $2\frac{1}{2}$ hours the total loss of activity was then four times as much as the gain of adrenaline. This experiment indicates that the methylation of noradrenaline proceeded quickly and was not limited by the time of incubation but by other factors

DISCUSSION

Blaschko's view (1939, 1942) that noradrenaline is the precursor of adrenaline has been substantiated by the experimental results presented in this paper Noradrenaline and adrenaline are both present in suprarenal glands. They are both released, in varying proportions, from the gland during splanchnic stimulation, which might suggest that noradrenaline is an end-product by itself. It has now been shown that it can be used by the suprarenal gland as precursor for the synthesis of adrenaline

For the methylation of noradrenaline by suprarenal extracts the presence of ATP appeared to be essential. No conversion took place in the absence of ATP. When noradrenaline was added some samples converted a proportion of the added amount as well as the amount initially present in the gland. In other samples only a moderate degree of conversion took place in spite of a large surplus of noradrenaline being available. Glands which had been subjected to prolonged splanchnic stimulation showed an increased capacity for the methylation of noradrenaline, while glands removed as quickly as possible under the most favourable conditions converted relatively small amounts

The conversion appeared to be completed within 1 hour (shorter periods have not been tested) during which the loss of total activity was negligible. If samples were, however, incubated for several hours a progressive loss in total activity was observed

Holtz and Kroneberg (1948) proposed epinine rather than noradrenaline as a precursor for adren-

aline on the ground that noradrenaline could be released from the suprarenal gland as an end-Recently (Holtz and Kroneberg, 1949) these authors have investigated the possibility of adrenaline synthesis by suprarenal extracts from phenylethylamine, tyramine, and oxytyramine Incubation of extracts for several hours with the addition of any of these three amines produced an increase in pressor activity which the authors believe to be most probably due to the formation of adrenaline It may, however, be due to various other reasons Firstly, the control was neither acidified nor boiled All the samples were shaken for several hours before the actual experiment was started in order to deplete the initial store This depletion probably continued in the control samples during the period of the experiment while in samples to which the different amines were added the activity of amine oxidase might have been inhibited Thus the stronger pressor activity could have been due not to a synthesis of adrenaline but to a prevention of its destruction

On the other hand, a synthesis of adrenaline, either via epinine or via noradrenaline, is considered by the authors In the light of the experiments reported in this paper the synthesis of adrenaline is unlikely because no ATP was added synthesis up to one stage before the end-product is The synthesis of epinine is unlikely possible because of its feeble pressor activity. The synthesis of noradrenaline is the most probable because it has a strong pressor activity and it may have been formed from oxytyramine during prolonged shaking In their concluding remarks Holtz and Kroneberg (1949) correct their previous view by putting forward the theory that in the suprarenals the chief mechanism of adrenaline synthesis proceeds from oxytyramine via noradrenaline to adrenaline A side mechanism, which would enable the suprarenals to use phenylethylamine or tyramine for adrenaline synthesis, needs further analysis by suitable methods Already in 1940 Vinet reported

experiments in which she incubated minced suprarenal tissue with hydroxytyramine and found a formation of what she believed to be adrenaline. For the estimation a colorimetric method was used, which, according to Euler does not distinguish between adrenaline and noradrenaline. Thus in Vinet's experiments also a formation of noradrenaline from hydroxytyramine might have taken place

The methods employed for the assays in my experiments were far from accurate. It must, however, be emphasized that they agreed, without exception, in the estimation of the relative activity of different samples. The results are further strengthened by the use of known mixtures of adrenaline and noradrenaline as standard solutions.

In a few samples the proportion of adrenaline to noradrenaline was changed in the opposite direction indicating the possibility of the existence of a mechanism for the demethylation of adrenaline resulting in a formation of noradrenaline. This has been suggested by Bacq and Fischer (1947) as one explanation for its presence in tissue extracts

SUMMARY

1 Suspensions of ground dogs' and cats' suprarenals are capable, during 1 hour's incubation at 37° C, of converting *nor*adrenaline to adrenaline

2 The presence of ATP is essential for the methylation of *nor* adrenaline

3 Glands removed after prolonged splanchnic stimulation have a higher methylating power than glands taken from a freshly killed animal

I wish to thank Dr L A Stocken, of the Department of Biochemistry, Oxford, for the supply of ATP used for these experiments, and Miss U Pardoe for her careful assistance I also wish to thank Dr M L Tainter for a supply of racemic noradrenaline

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FORMATION OF ADRENALINE FROM NORADRENALINE IN THE PERFUSED SUPRARENAL GLAND

BY

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Observations on the secretion of adrenaline from the perfused suprarenal gland of the dog have been described by Bülbring, Burn, and de Elío (1948) These experiments were carried out before the publication of work by various authors, Schümann (1948), Bülbring and Burn (1949a and b) and v Euler and Hamberg (1949), showing that the suprarenal medulla contains noradrenaline as well as adrenaline, and no tests were used which would have distinguished between the two substances The numerous estimations were made with isolated loops of rabbit intestine, comparing the samples with adrenaline The main outcome was to show that the liberation of adrenaline-like material in the blood leaving the gland was modified by the presence of adrenaline in the perfusing blood. When this was very small in amount, stimulation of the splanchnic nerve liberated very little adrenaline-like material in the venous blood When the experiment was performed with progressively larger amounts of adrenaline in the perfusing blood, stimulation of the splanchnic nerve liberated progressively larger amounts of adrenaline-like material until an optimum concentration for adrenaline in the perfusing blood was reached A further increase of adrenaline in the perfusing blood beyond this optimum produced a steady decline in the amount of adrenaline-like substance liberated by splanchnic stimulation

The work had, however, been begun with the object of studying the formation of adrenaline by the isolated gland, and in the experiments in which less than 1 µg adrenaline per ml was present in the perfusing blood, an increase in the amount of adrenaline-like material in the system was always observed. Meanwhile West (1947), working in the pharmacological laboratory in Edinburgh, had shown that the rat uterus was much more sensitive to adrenaline than to noradrenaline. When used as described by de Jalon, Bayo, and de Jalon (1945) this test can be used to estimate adrenaline in a mixture of the two drugs (see Gaddum, Peart, and Vogt, 1949)

We therefore carried out a series of five perfusion experiments in which we estimated adrenaline and noradrenaline separately throughout each experiment, and we now give a brief description of the results which were obtained

METHODS

The experiments were performed as already described by Bulbring, Burn, and de Elio (1948), the right gland being first removed in order to determine the content of adrenaline and noradrenaline per g, this was assumed to be the content of the left gland at the beginning of the perfusion. All venous blood from the perfused gland was collected in a series of samples, and after the cells had been spun off, the plasma was tested. The left gland was tested at the end of the perfusion. By comparisons with adrenaline on the rabbit intestine a figure was obtained which we took as the sum of adrenaline + noradrenaline. By comparisons with adrenaline on the rat uterus we obtained a figure which we took as the amount of adrenaline only

RESULTS

Perfusion was carried out for periods which varied in different experiments from 2 hr 7 min to 3 hr 25 min. The rate of flow through the gland diminished during the course of the perfusion. The following are the details of an experiment in which the flow was good throughout, starting at the rate of 12 c c /min and being 2 25 c c /min at the termination 2 hr 19 min later.

Experiment No 1

Right gland Total 2,391 μ g or 1 65 mg/g Adrenaline 1,162 μ g 0 8 mg/g (by difference) Noradrenaline 1,229 μ g 0 85 mg/g The left gland weighed 1 6 g Its active content at the beginning of the perfusion was assumed to be the same per g as that of the right gland, hence

Left gland Total 2,620 μg Adrenaline 1,260 μg Noradrenaline 1,360 μg At the end of the perfusion the contents of the left gland were found to be

Total 2,062 μg Adrenaline 1,762 μg Noradrenaline 300 μg

During the perfusion the splanchnic nerve was stimulated continuously for 1 hr, and at the end of this period noradrenaline was added to the perfusing blood so that the concentration present in terms of l-noradrenaline was 54 μg /cc During the rest of the perfusion 144 cc of blood passed through the gland so that the amount of noradrenaline added to the system in this way was 777 μg In the venous blood from the perfused gland there was found

Total 1,073 μg Adrenaline 307 μg Noradrenaline 766 μg

The following balance could therefore be drawn At beginning At end Adrenaline in gland 1,260 μ g 1,762 μ g 307 μ g Noradrenaline in gland 1,360 μ g 300 μ g

,, added to blood 777 μ g , in venous blood

766 μg
3,397 μg
3,135 μg

The figures for the totals at the beginning and at the end show fairly close agreement, there being a loss of less than 8 per cent. There was, however, a large loss of 1,071 μ g noradrenaline and a large gain of 809 μ g adrenaline. The evidence supports the view that during the perfusion noradrenaline was converted to adrenaline.

Of the four other experiments one was unsatisfactory because of the poor rate of flow through the gland. There was little change in the total contents of the system though the stimulation of the splanchnic nerve liberated both adrenaline and some noradrenaline in the venous blood. The balance was as follows

Experiment No 2

A	t beginning	At end
Adrenaline in gland	$1,215 \mu g$	415 μg
,, in venous bloo	d	821 μg
Noradrenaline in gland	0 μg	85 μg
,, added to blood	155 μg	
,, in venous blood		244 μg
		
	1,370 μg	1,565 μg

The total adrenaline was almost identical in amount at the end as at the beginning (1,236 μ g as compared with 1,215 μ g) though two-thirds of it appeared in the venous blood. The total noradrenaline rose from 155 μ g to 329 μ g. This change was an increase

of 174 μg on an initial total of 1,370 μg , that is to say, it was 127 per cent. The three remaining experiments were satisfactory. Since the results were similar to those in Exp. 1 they have been summarized together in Table I

TABLE I
CHANGES OBSERVED DURING PERFUSIONS

Exp	Total		Adrenaline		<i>Nor</i> adrenaline	
Ехр	μg	%	μg	%	μg	%
1 3 4 5	-262 +235 +226 -503	-8 +22 +16 -16	+809 +315 +292 +315	+64 +40 +29 +18	- 1,071 - 80 - 66 - 810	-51 -26 -17 -61

The first point shown in the Table is that in each experiment there was a gain in adrenaline, and in each there was a loss in noradrenaline. Even in the two experiments in which there was a net loss, there was nevertheless a gain in adrenaline, and in those in which there was a net gain this was exceeded by the gain in adrenaline. The changes have also been expressed as percentages of the amounts originally present. These percentages are large enough to show that the absolute changes are not so small as to be within the error of the biological methods used

When each experiment is taken singly there is some correspondence between the loss of nor-adrenaline and the gain in adrenaline in experiments. Nos 1 and 5, but not in experiments Nos 3 and 4, in which the recorded loss of noradrenaline was small. In experiments 3 and 4 the glands contained adrenaline only, both at the beginning and at the end of perfusion, while in experiments 1 and 5 the change shown in Table II took place. In these

TABLE II CONTENTS OF GLAND

	At beginning		At	end -	
No of exp	% Adr	% Nor	% Adr	% Nor	
1 5	48 73	52 27	86 96	14 4	

experiments both glands contained initially a considerable amount of *nor*adrenaline which at the end of the perfusion was very small. This finding also suggests that *nor*adrenaline was converted into adrenaline.

DISCUSSION

The experiments furnish evidence that when the dog's suprarenal gland is isolated from all other tissues and is perfused with heparinized blood from a pump, noradrenaline is methylated to form adrenaline in the course of the perfusion. A change consistent with this conversion was observed in each of four satisfactory experiments. In a fifth experiment in which the blood flow was poor there was no evidence of this change. The absence of conversion in this experiment added weight-to the evidence of conversion in the others.

In each experiment the right suprarenal was analysed, without having been perfused, as a control, and in three of the five glands so examined noradrenaline was found present as well as adrenaline. In these the noradrenaline constituted respectively 14, 28, and 52 per cent of the total. In two of the glands, however, we found only adrenaline, and in glands taken from two other dogs also we found only adrenaline. For this reason noradrenaline was added to the perfusing blood in the perfusion experiments

These results agree with the evidence of Bulbring (1949) that minced suprarenal tissue transforms noradrenaline to adrenaline. She observed that this process of methylation was much more active when the glands had first been depleted by a period of splanchnic stimulation. The splanchnic nerve was stimulated in experiments 1 and 3 of the perfusions, and the percentage increase in adrenaline was

greater in these experiments than in the others in which the splanchnic nerve was not stimulated

SUMMARY

- 1 The dog's suprarenal gland may contain not only adrenaline, but also *nor*adrenaline. Out of seven glands three were found to contain both these substances
- 2 When the gland was perfused with heparinized blood, noradrenaline being added to this blood, an increase in the amount of adrenaline was observed coinciding with a decrease in the amount of noradrenaline. The conversion of noradrenaline to adrenaline is indicated

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STUDIES IN THE CHEMOTHERAPY OF TUBERCULOSIS PART V THIOSEMICARBAZONES AND RELATED COMPOUNDS

BY

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It has been reported (Domagk, Behnisch, Mietzsch, and Schmidt, 1946, Domagk, 1948) that some thiosemicarbazones of cyclic aldehydes and ketones show antituberculous activity in vitro instances, activity in vivo was also demonstrated in experimental animals, and certain compounds have been used in the treatment of lupus and pulmonary tuberculosis (Moncorps and Kalkoff, 1947, Domagk, 1948, Kuhlmann, 1948) with results which are claimed to be encouraging We have undertaken a systematic investigation of derivatives of thiosemicarbazide both in vitro and in vivo, using methods which have been described in earlier papers in this series (Martin, 1946, Hoggarth and Martin, 1948a) The compounds which have shown most interest are all thiosemicarbazones of monosubstituted benzaldehydes, the general formula of which may be written as follows

The test method consisted of the infection of mice by the intravenous route, and their treatment by drugs administered orally twice daily by syringe and catheter at doses ranging downwards from the maximum tolerated. The results are presented in the form used in preceding papers

- (i) Substitution in the aryl residue —Thiosemicarbazones of variously substituted benzaldehydes are listed in Tables I and II The parent aldehydes in Table I carry one substituent. In some instances the same substituent has been placed in the three possible positions with respect to the thiosemicarbazone group. The compounds in Table II have two substituents in the aryl ring
- (ii) Thuosemicarbazones of heterocyclical aldehides—The results are given in Table III Com-

TABLE I

ANTITUBERCULOUS TESTS ON SOME THIOSEMICARBAZONES OF MONOSUBSTITUTED BENZALDEHYDES

Doses given twice daily by syringe and catheter

Compounds of the form

NH2

Ar CH N NH C

No	Ar	Dose (mg per 20 g mouse)	Increased mean survival time (days)	Increase required for significance (days)
5669	phenyl	0 25 0 5 0 5	+01 +11 +27	} 17 30
5670	p-chlorophenyl	0.25 0.5 0.5	-03 +21 +33	} 17 30
6056	p-nitrophenyl	0 5 1 0 0 5 1 0	+43 +67 +72 +87	} 17 } 22
6267	nı-nıtrophenyl	0 1 0.25	+0 1 -0 3	} 17
5704	o-nitrophenyl	0 25 0 5 1 0	-03 +09 -01	} 17
6198	p-aminophenyl	10	+33	1 4
6087	p-methylamino- phenyl	1 0 3 0	+4 1 +2 4	} 16
6147	p-ethylamino- phenyl	0 25 0 5	+3 2 +3 5	} 14
5672	<i>p</i> -dimethyl- aminophenyl	1 0 1 5 2 0	+47 +51 +65	16

TABLE I-contd

IABLE I—wind						
No	Ar	Dose (mg per 20 g mouse)	Increased mean survival time (days)	Increase required for significance (days)		
6148	p-methylethyl- ammophenyl	0 25 0 5	+22 +29	} 14		
5916	p-diethylamino- phenyl	0 25 0 5	+09 +34	} 19		
5961	N-p-pyrroli- dinophenyl	0 25 0 5	0 +57	} 22		
6473	p-di-β-chloro- ethylamino- phenyl	0 1 0 25	-04 +05	} 17		
6478	p-ethyl-β- chloroethyl- ammophenyl	1 0	+22	1 2		
6082	p-hydroxy- phenyl	10 0 10 0 10 0 20 0	+84 +81 +108 +112	2 8 1 3 2 0		
5958	o-hydroxy- phenyl	5 0 10 0	+03	1 4 1 6		
6057	p-anisyl	2 5 5 0 5 0	+61 +84 +101	1 4 2 2 1 2		
6083	m-anisyl	1 0 2 0 5 0	13 15 45	} 14		
6253	o-anisyl	0 25 0 5	0 +07	} 14		
6524	p-phenetidyl	20	+45	1 4		
6772	p-isopropoxy- phenyl	1020	+42 +56	} 14		
6462	p-methylmer- captophenyl	0 25 0 5	+28 +47	} 14		
8574	<i>p</i> -methylsul- phonylphenyl	5 0*	+100	3 3		
8388	p-ethylsul- phonylphenyl	5 0 10 0 10 0	+12 7 +11 6 +9 8	} 27		
8580	<i>p-n</i> -propylsul- phonylphenyl	10 0	+93	28		
6463	p-thiocyano- phenyl	05	+4.2 +5 6	} 14		

^{*}This compound proved toxic at this dose, and ten of the twenty mice present originally in the treated group died in the first few days of the experiment

pound No 6060 is the thiosemicarbazone corresponding to 5-nitrofurfural semicarbazone ("Furacin") The latter was also tested under the same conditions (doses 0 5 and 1 0 mg per 20 g mouse) and was inactive *

(111) Aliphatic thiosemicarbazones—Thiosemicarbazones derived from a few aliphatic aldehydes (heptaldehyde, cinnamaldehyde, p-dimethylamino-

TABLE II

ANTITUBERCULOUS TESTS ON SOME THIOSEMICARBAZONES OF DISUBSTITUTED BENZALDEHYDES

Doses given twice daily by syringe and catheter Compounds of the form

Ar CH N NH C

No	Ar	Dose (mg per 20 g mouse)	Increased mean survival time (days)	Increase required for significance (days)
5887	2 4-dinitro- phenyl	0 5 1 0 2 0	+05 +08 +19	} 14 16
6465	3-nitro-4- dimethyl- aminophenyl	0 25 0 5	+01	} 10
6464	3-nitro-4- diethyl- aminophenyl	0 25 0 5	+0 4 +1 4	} 12
6475	2-chloro-4-dı-β- chloroethyl- amınophenyl	2 0	+08	17
6468	4-ethylamino- m-tolyl	0 1 0 25	+1 1 +1 0	} 13
6476	4-dı-β-chloro- ethylamıno- o-tolyl	1 0 3 0	-19 +13	} 17
6222	4-hydroxy-m- anısyl	8 0	+06	1 4
6255	3 4-dimethoxy- phenyl	1 0 2 0 2 0	+1 4 +2 9 +3 1	} 14
6266	4-ethoxy-m- anisyl	50	+53	1 4
6197	3 4-methylene- dioxyphenyl	0 5 1 0	+1 9 +3.2	} 14

^{*}It has been shown recently that "Furacin" has no therapeutic effect in tuberculous guinea pigs (Wolinsky, E. Wetzel, V, and Steenken, W., 1949, Proc. Soc exp Biol Med., 70, 483)

TABLE III

ANTITUBERCULOUS TESTS ON SOME THIOSEMICARBAZONES OF HETEROCYCLICAL ALDEHYDES

Doses given twice daily by syringe and catheter

Compounds of the form

No	R	Dose (mg per 20 g mouse)	Increased mean survival time (days)	Increase required for significance (days)
6060	2-(5-nitrofur- furyl)	1 0 2 0	$-16 \\ -32$	} 17
6116	2-quinolyl	2 5 5 0	+09 +11	} 12
6099	4-quinolyl	1 0 5 0	+1 6 +6 2	} 12

cinnamaldehyde, and dextrose) were all without any activity and results are not given in detail

- (iv) Substitution in the thiosemicarbazone group— The hydrogen atoms of the thiosemicarbazide residue—1 e., those on nitrogen atoms N^3 and N^4 , and also that on the sulphur atom in isothiosemicarbazide ($NN C(SH)NH_2$)—were replaced by alkyl groups, singly or in pairs. The parent aldehyde for most of these derivatives was p-dimethylaminobenzaldehyde or p-anisaldehyde. Results with these compounds are reported in Table IV a and b
- (v) Ketone thiosemicarbazones—Some thiosemicarbazones derived from aliphatic and aromatic ketones (e g, acetophenone, p-methoxyacetophenone, 5-diethylaminopentane-2-one) were found to be inactive
- (vi) Benzaldehyde semicarbazones and benzalaminoguandines —No activity was found in the semicarbazones of benzaldehyde, p-hydroxy, p-methoxy-, or p-dimethylaminobenzaldehyde, nor in the benzalaminoguanidines derived from the two last-named aldehydes
- (vii) Related compounds—The inactivity of the compounds mentioned in paragraph (vi) above indicated that the thioureido portion of the thiosemicarbazide group was essential. We have therefore examined other compounds in which the spatial relationship between an aryl nucleus and a thioureido group was similar to that in the benzaldehvde thiosemicarbazones. These compounds (21 in

TABLE IV

ANTITUBERCULOUS. TESTS ON SOME SUBSTITUTED THIO SEMICARBAZIDE DERIVATIVES OF p-DIMETHYLAMINOBENZ-ALDEHYDE

Doses given orally twice daily by syringe and catheter

- (a) Compounds of the form

No	Subs	stituent N ⁴	s on	Dose (mg per 20 g mouse)	Increased mean survival time (days)	Increase required for significance (days)
5873		CII				
38/3	H	CH ₃	H	1 0 2 0	$-0.2 \\ +0.3$	} 19
5874	H	C ₂ H ₅	H	5 0	+16	19
5875	н	(n) C ₃ H ₇	Н	1 0 2 0	+07 +07	} 12
5876	Н	(180) C ₃ H ₇	Н	7 0	-08	19
5877	Н	(n) C ₄ H ₉	н	5 0 10 0 10 0	-15 -09 +01	} 22 19
5878*	Н	(150) C ₄ H ₉	Н	1 0 5 0 5 0 2 5 5 0	+25 +59 +57 +33 +85	} 22 19 } 22
6258*	Н	(sec) C ₄ H ₉	Н	-10 20	-02 -10	} 14
6067	СН	CH ₃	Н	10	+10	1 6
6068	CH ₃	C ₂ H ₅	Н	0 5 1 0	+1 6 +0 5	} 16
6069	СН3	(150) C ₃ H ₇	Н	0 5	+04	1 6
6070	СН3	(n) C ₁ H ₉	Н	10 0	+19	1 6
6290*	Н	СНэ	CH ₃	0 5 1 0	-0 8 -0 5	} 14

Compounds analogous to Nos 5878 6258 and 6290 but prepared from p-anisaldehyde in place of p-dimethylaminobenzaldehyde were inactive.

TABLE IV-contd

(b) Compounds of the form

(4)
NR

(CH₃)₂N

CH N NR C

SCH₃

No	Substit N ²	uents on	Dose (mg per 20 g mouse)	Increased mean survival time (days)	Increase required for significance (days)
6085	Н	Н	0 5 1 0	-03 +02	} 16
6080	Н	СН,	05	0 -07	} 14
6081	Н	(150) C ₃ H ₇	1 0 2 0	-0 4 -0 4	} 15
6086	CH ₃	CH ₃	0 5 1 0	-07 +04	} 14

all) fall into the first six classes listed below. The last category (phenyl thioureas) represents the simplest possible combination of an aryl nucleus and a thioureido group. All these compounds were completely inactive.

Ar CH N NH CS NH₂

(Benzaldehyde thiosemicarbazones)

Ar CO NH NH CS NH2

1-Benzoylthiosemicarbazides

Ar SO₂NH NH CS NH₂

1-Benzenesulphonylthiosemicarbazides

Ar CH N NH CS NH N CH Ar

Dibenzalthiocarbhydrazides

Ar NH CS NH CS NH2

Phenyldithiobiurets

Ar NH C(NH) NH CS NH:

Phenylguanylthioureas

Ar CO NH NH CS NH CO Ar

1 4-Dibenzoylthiosemicarbazides

Ar NH CS NH.

Phenylthioureas

(Ar = an aryl residue usually with a para substituent which was either Cl, NO₂, HO, CH₃O, (CH₃)₂N, CH₃, or NH₂ SO₂)

TABLE V

FURTHER ANTITUBERCULOUS TESTS ON THE MORE ACTIVE THIOSEMICARBAZONES OF MONOSUBSTITUTED BENZALDEHYDES Drugs (mixed with powdered food) administered to groups of 20 mice for the first fourteen days of the test. Compounds of the form

No	R	Estimated daily intake (mg) of drug per 20 g mouse	Observed mean survival time (days)	Increased mean survival time (days)	Increase required for significance (days)	First death (days)	Last death* (days)
5672	N(CH ₃) ₂	1 5 2 0	20 7 24 2	3 8 6 5	1 5 2 1	17 18	26 29(5)
6057	OCH3	5 0 10 0 5 0 5 0	23 8 25 9 20 7 25 3	6 1 8 2 3 8 6 6	} 21 {	21 14 16 17	29(1) 29(3) 25(2) 33
6082	ОН	10 0 20 0 2 0 5 0 10 0	29 6 30 0 22 4 27 2 30 5	10 8 11 2 2 8 7 6 10 9	\begin{cases} 20 \\ 30 \\\\\\\\\\\\\\\\\\\\\\\\\\\\\\	19 20 18 20 22	35(2) 35(5) 30 31 33
8388	SO ₂ C ₂ H ₈	2 0 5 0 10 0	32 7 34 8 35 4	13 1 15 2 15 8	} 30 {	25 26 28	40 50 50

^{*} Figures in parentheses give number of survivors at the termination of the experiment. In all the above experiments all control mice were dead by the 22nd day except for a single mouse which survived to the 27th day in one test

(viii) Further comparisons of active compounds— Examination of Table I reveals that highest activity is shown by the thiosemicarbazones of p-hydroxy-benzaldehyde (6082), p-anisaldehyde (6057), and the group of p-alkylsulphonylbenzaldehydes (8388, 8574, 8580) With all these compounds increases up to ten or twelve days in the mean survival time have been recorded, and it was felt desirable to compare these compounds directly one with another

For this purpose we have preferred to administer the drugs mixed with the powdered food over a period of fourteen days, as previously described (Hoggarth and Martin, 1948b) We find that, on the whole, the effects observed with the two methods of administration (when the total daily intake of drug is the same) do not differ greatly from one another The results are given in Table V

The toxicities of the compounds in Table V are of interest in connexion with their therapeutic effects. In general, the highest doses quoted in this table were the highest found to be tolerated without serious toxic effects by uninfected mice. We have not attempted to determine median lethal doses, but the observations recorded in Table VI were made.

TABLE VI
TOXIC EFFECTS OF THIOSEMICARBAZONES NOS 5672, 6057, 6082, 8388

Drugs given by syringe and cathether twice daily to groups of twelve mice for three weeks

No	Dose (mg per 20 g)	Effect
5672	1 0 2 0	None Growth slightly retarded but no deaths
	40	Failure to gain weight (4 deaths)
6057	4 0 6 0 8 0 10 0	None Growth retarded but no deaths
6082	10 0	None
8388	4 0 6 0 8 0 10 0	None Growth slightly retarded but no deaths

From these limited observations, 6082 appears to be the least toxic, and in another experiment mice were given food containing 0.25% of this substance (estimated daily intake of drug 10 mg per 20 g) for 7 weeks without obvious toxic effects. When

this concentration of 8388 was added to the food, the animals at first refused to eat it but later did so, without apparent harm, for 120 days

DISCUSSION

Our investigation of thiosemicarbazones and related compounds has shown that marked activity against an acute infection with Mycobacterium tuberculosis in mice is limited to the thiosemicarbazones of substituted benzaldehydes or heterocyclical aldehydes Substitution of the hydrogen atoms of the thiosemicarbazide residue by alkyl groups usually abolishes activity, and the few thiosemicarbazones derived from alkyl-substituted thiosemicarbazides which are active are usually less Activity has not so than the parent compound been found amongst the many closely related compounds examined The relationship between activity and the type and position of substituents in the aromatic nucleus of benzaldehyde thiosemicarbazones may be inferred from Tables I and II For highest activity a para substituent is necessary, though a few substituents in the meta position confer activity High activity is found with alkylsulphonyl, nitro, amino, hydroxy, mercapto, and thiocyano groups in the para position, but only low activity with a para chloro group Alkylation of the para hydroxy group does not result in significantly higher activity Amongst the p-aminobenzaldehyde thiosemicarbazones the peak of activity is shown by the dimethylamino compound We have examined three thiosemicarbazones of p-alkylsulphonyl benzaldehydes, all of which are highly active, the ethylsulphonyl compound is possibly the most active The methylsulphonyl compound is more toxic than the other two Introduction of a second substituent in the aryl residue usually reduces activity greatly The activity of quinoline-4-aldehyde thiosemicarbazone is within the range of activity of the benzaldehyde thiosemicarbazones

The response observed with the most active compounds of this type (Nos 6057, 6082, 8388, 8574, and 8580) under the particular conditions of our routine test was better than that obtained with any other synthetic substance (including p-aminosalicylic acid) which we have yet examined. When the drugs were given mixed with the food for the first fourteen days only of the test, it has proved possible with most of these compounds to keep all treated animals alive until after the death of the last control animal. The treated animals did eventually die with marked tuberculous lesions of the lungs

The activity in vitro of all the compounds mertioned above was determined by our usual method (Hoggarth and Martin, 1948a), but the results are

not given here in order to save space. We agree with the general statement of Domagk and his co-workers that the compounds with most activity in vitro in this series will inhibit growth of the tubercle bacillus at concentrations of 1 50,000-1 100,000 In our *in vitro* test we commonly find that the concentration at which growth is equal to controls is not less than 1/9 or 1/27 of the concentration for complete inhibition—e g, No 6082 will completely inhibit growth at 1 81,000 but has no inhibitory effect at 1 2,000,000 With certain compounds of this series—e g, Nos 6056, 6099, 6255, 6266, 8388—partial growth took place even at 1 1,000, the highest concentration tested, but inhibition of growth could be detected at a concentration of 1 729,000 or 1 2,000,000 We believe this unusually wide range of partial activity to be a real phenomenon, as it has been observed repeatedly with the substances mentioned and also occasionally with substances having no therapeutic action Many compounds of high activity in vitro were devoid of activity in vivo

The substances tested clinically by the German workers may be identified by the key given by Domagk (1947) These correspond to our numbers 8388, 6057, and the acetyl derivative of our number 6198 We find that the thiosemicarbazone of *p*-ethylsulphonylbenzaldehyde (8388) is superior in

its therapeutic effect to any of the other compounds of its class which we have examined. This is a reflection of its marked persistence in the blood stream (Spinks, 1949)

SUMMARY

Antituberculous activity in mice has been investigated in certain derivatives of thiosemicarbazide and related compounds. After a study of about one hundred such compounds, therapeutic activity has been found to be confined to thiosemicarbazones of substituted benzaldehydes and heterocyclical aldehydes

Our thanks are due to Dr N Barton of these laboratories for samples of phenyldithiobiuret compounds originally prepared by him for antimalarial test

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THE ESTIMATION OF SOME THIOSEMICARBAZONES AND THEIR BLOOD CONCENTRATIONS IN EXPERIMENTAL ANIMALS

RY

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Thiosemicarbazones of some aromatic aldehydes have been shown to possess appreciable antituber-culous activity (Domagk, Behnisch, Mietzsch, and Schmidt, 1946, Domagk, 1948, Hoggarth, Martin, Storey, and Young, 1949) Among the compounds described by Hoggarth *et al* (1949) the most promising were *p*-anisaldehyde thiosemicarbazone (6057, I, R = CH₃O), *p*-hydroxybenzaldehyde semicarbazone (6082, I, R = OH) and *p*-ethylsulphonylbenzaldehyde semicarbazone (8388, I, R = C H₄SO₂) The methods described here were

R—CH=N—NH—CS—NH₂

$$6057 R = CH_3O (I)$$

$$6082 R = OH$$

$$8388, R = C_2H_8SO_2$$

developed so that the absorption and excretion of these three compounds could be studied in relation to their therapeutic effects in mice

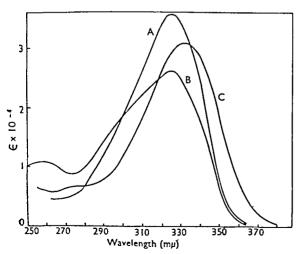


Fig 1 —Absorption spectra of 6057 (A), 6082 (B) and 8388 (C) in chloroform Ordinates $\epsilon \times 10^{-4}$, abscissae wavelength (m μ)

EXPERIMENTAL SECTION

It is known that certain unsaturated thiosemicarbazones show intense absorption of ultraviolet light (Heilbron, Johnson, Jones, and Spinks, 1942) The absorption spectra of the three compounds in chloroform were therefore examined by means of the Beckman photoelectric spectrophotometer (Fig 1). They were quite suitable for the purpose of estimation provided that a method of extraction from biological fluids could be devised. It was found that 6057 and 8388 could be extracted by the same simple procedure but that 6082 could not Descriptions of the methods and of their use in animal experiments follow.

1 Estimation of p-anisaldehyde thiosemicarbazone (6057) and p-ethylsulphonylbenzaldehyde thiosemicarbazone (8388) in blood

Reagents

- 1 0 2M-disodium hydrogen phosphate, preserved by adding a trace of chloroform
- 2 BP chloroform
- 3 0 2 g /100 ml stock solution of 6057 or 8388 in methanol (stable in the dark)
- 4 1 mg/100 ml standard solution, prepared Just before use by diluting the stock solution with water

Procedure

Pipette 2 ml of blood into 4 ml of 0 2M-disodium hydrogen phosphate in a 60 ml glass-stoppered bottle Add 40 ml of chloroform and shake for 5 min. An emulsion rarely forms, and can always be resolved by centrifuging the bottle. Withdraw most of the lower layer and clarify it by filtration through Whatman No. 1 or similar semi-fine paper, or by shaking it in a clean dry bottle with about half a gram of anhydrous sodium sulphate, A.R. Transfer it to a 10 cm silical spectrophotometer-cell, and read the optical density at $325 \text{ m}\mu$ (6057) or $332 \text{ m}\mu$ (8388) against a blank. Let the reading be "a" Prepare the blank and a standard at the same time as the unknown by substituting 2 ml of distilled water, and 2 ml of the standard solution for blood in

the above procedure Read the standard also against the blank Let the reading be "b" Then the concentration of 6057 or 8388 in the unknown is a/b mg /100 ml Normal blood gives a reading equivalent to about 0.05 mg /100 ml, which must be subtracted from the experimental results

Notes

It was established that the graph of concentration against optical density was a straight line a single standard was therefore adequate Disodium hydrogen phosphate was added because previous experience with similar methods (Spinks, 1946) had shown that the extraction of interfering materials from blood was reduced when the blood was buffered to At first this caused difficulty weak alkalinity because the disodium hydrogen phosphate used contained mercuric chloride as preservative Extraction of 6057 was completely prevented, presumably because it formed a solvent-insoluble mercury salt When a trace of chloroform was substituted for mercuric chloride recovery from water and blood The recovery from was quantitative (Table I)

μg a	μg added		ound	% recovery		
6057	8388	6057	8388	6057	8388	
2 4 6 - 10 14 20	5 10 15 25 35 50	1 83 3 97 5 88 9 50 13 3 20 0	4 96 9 25 15 1 25 0 34 3 46.2	91 99 98 95 95 100	99 92 101 100 98 92	

plasma and urine was also quantitative. However, concentrations of 6057 in urine were later found to be very low, so that its presence had to be confirmed, and its amount assessed, by constructing a full absorption spectrum of each extract. Concentrations of 8388 in urine were much higher, and the method described could be used without modification.

 Estimation of p-hydroxybenzaldehyde thiosemicarbazone (6082) in plasma

Reagents

- 1 4M-sodium dihydrogen phosphate
- 2 BP chloroform
- 3 1 g /100 ml sodium carbonate
- 4 and 5 Solutions of 6082 as described for 6057 and 8388

Procedure

Pipette 2 ml of plasma (which must not be contaminated by haemolysis) into a bottle Add 2 ml of 4Msodium dihydrogen phosphate and 40 ml of chloroform Shake vigorously for 5 min and filter the lower layer into a measuring cylinder Transfer an aliquot of 30 ml to a dry bottle Add 8 ml of 1 per cent sodium carbonate and shake vigorously for 3 min Decant the upper layer into a test tube, centrifuge it for 5 min, and transfer it Read the optical to a 2 cm spectrophotometer cell density ("a") against a blank at 330 mµ and a standard are prepared by substituting 2 ml of water and 2 ml of a 1 mg/100 ml standard solution of 6082 for plasma in the above procedure If the reading of the standard is "b" the concentration of 6082 in the unknown is Fa/b mg/100 ml, where F is a recovery factor derived from data such as those of Table III

Notes

Only a trace of 6082 was extracted from water or blood under the conditions used for 6057. This was shown to be due to two effects—first, 6082 was a stronger acid (pKa about 95) than its phenolic structure had suggested (Table II), and second, it could not be fully extracted even at low pH values unless salted out by a high concentration of buffer (Table II). Further, when a pH of 48 and a high

TABLE II effect of ph of added buffer, and molarity of added NaH $_2$ PO $_4$ on the extraction of 6082 from water into chloroform

pH of 0 2 M-buffer	% Extraction	Molarity of NaH ₂ PO ₄ (pH 4 8)	% Extraction
5 0 6 0 7 0 8 0 8 9 10 6 10% Na ₂ CO ₃	48 45 46 41 32 6 5 0	4 0 2 0 1 0 0 5 0 2	101 88 70 60 52

buffer concentration were used in the extraction of blood very large amounts of interfering material, equivalent to several mg of 6082/100 ml, were extracted, and even then the recovery of 6082 was only about 20 per cent. Consequently, piasma was analysed instead. Interfering material was again removed, but in less amount, and when the 6082 was re-extracted into 1 per cent sodium carbonate (cf. Table III) the blank was reduced almost to zero. The recovery of 6082 from plasma was incomplete but adequate (Table III). The recovery from urine was theoretical, but 6082, like 6057, reaches only low concentrations in urine, and full absorption spectra of extracts must be constructed.

TABLE III
RECOVERY OF 6082 FROM PLASMA

	Four	%	
Added, μg	1st extraction (CHCl ₃)	Re-extraction (1% Na ₂ CO ₃)	Recovery (Na ₂ CO ₃)
0 4 10 20 30 40 0 20 40	6 17 9 75 14 0 19 3 26 2 32 4 4 25 18 9 39 0	0 07 3 00 7 24 14 9 23 2 31 2 0 12 8 29 0	73 72 74 77 78 64 72
		Mean	73

A disadvantage of analysing 6082 in plasma was that much data had already been obtained on the concentration of 6057 in blood. However, the validity of comparing blood with plasma concentrations in this series is indicated by the virtual identity of concentrations of 6057 in blood and plasma and by the fact that concentrations of 8388 in plasma are about 70 per cent of those in blood (results not shown). Another important point is that the major difference between the compounds is in rate of elimination from the blood, its apparent value could hardly be affected by analysing plasma instead of blood.

3 Animal Experiments

(a) Mice —Each compound was given to groups of 4 mice in doses of 500 mg/kg, administered orally

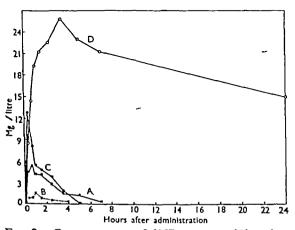


Fig 2—Concentrations of 6057 in mouse (A) and rat (B) blood, 6082 in mouse plasma (C) and 8388 (D) in mouse blood after the oral administration of 500 mg/kg Ordinates Milligrams per litre Abscissae Hours after administration

by syringe and blunt needle as a 2 per cent aqueous dispersion. Groups were killed at intervals after dosing by withdrawing heart blood under heavy chloroform anaesthesia. Blood from the 4 animals of a group was pooled for analysis. The concentrations found are shown in Fig. 2, which also includes the results of a similar experiment on 6057 in rats.

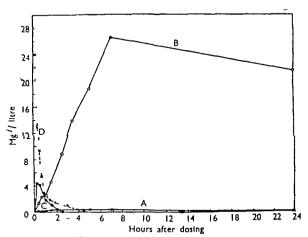


FIG 3—Concentrations of 6057 (A) and 8388 (B) in blood, and 6082 (C) in plasma, after oral doses of 250 mg/kg in rabbits. Each graph is the average of three experiments. Concentrations of 6037 in blood (D) after 1 v administration of 40 mg/kg to a single rabbit are also shown. Ordinates. Milli grams per litre. Abscissae. Hours after administration.

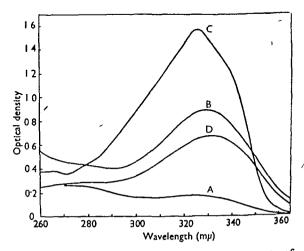


FIG 4—Absorption spectra of chloroform extracts of the first day s urine from rabbits receiving 6057 (A) and 8388 (B), and rats receiving 6057 (C), and of a 54 hour-blood sample (D) from a rabbit receiving 8388 Ordinates Optical density Abscissae Wavelength (mμ)

(b) Rabbits — Each compound was administered orally to rabbits as a 10 per cent aqueous dispersion, given by syringe and rubber catheter in doses of 250 mg/kg 6057 was also administered intravenously as a 2 per cent dispersion in a dose of 40 mg/kg Rabbits were bled from a marginal ear vein at intervals after dosing. The results are shown in Fig. 3. The identity of 8388 in a sample of rabbit blood taken 54 hours after dosing was confirmed spectrophotometrically (Fig. 4). 8388 could be detected in rabbit blood under these conditions for about a week.

Excretion of the three compounds in urine was examined using some of the above rabbits. Fig 4 shows the absorption spectrum of an extract of the first day's urine of a rabbit receiving 6057. It was estimated from such curves that the amount of 6057 excreted in the urine did not exceed 1 per cent of that administered (Fig 5), 6082 behaved similarly

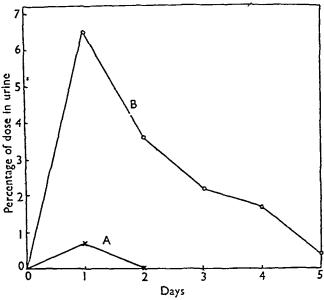


FIG 5—Excretion of 6057 (A) and 8388 (B) in the urine of typical individual rabbits after oral doses of 250 mg/kg Ordinates Percentage of dose in urine Abscissae Days

Rats excreted about 2 per cent of an oral dose of 500 mg of 6057/kg. Here the smaller urine volume permitted definite identification of the excreted material (Fig. 4). 8388 was readily identified even in rabbit urine (Fig. 4). Its behaviour was in sharp contrast to that of 6057 or 6082, since larger amounts appeared in the urine, and excretion continued over several days (Fig. 5). However, only about 10–14 per cent of the amounts administered to three rabbits were accounted for in the urine. Unfortunately, the analytical methods could not be applied to faeces, but it is improbable from analogy

with other compounds of similar simplicity that much material was lost in the faeces. It may reasonably be assumed that most of each compound was degraded in the body (cf. Discussion)

Discussion

The most important finding is that whereas 6057 and 6082 are rather rapidly eliminated, and attain only low blood or plasma concentrations, 8388 is very persistent and attains high blood concentrations. The marked difference between 8388 and the other two compounds is particularly evident when the areas beneath the blood concentration-time curves are measured. The figures for mice, expressed in arbitrary units, are 6057, 24, 6082, 31, 8388 (to 72 hrs. after dosing) 154

The results are in excellent agreement with the antituberculous properties of the three compounds, since 8388 is the least active in vitro (Martin, 1948) and the most active in vivo (Hoggarth et al, 1949). It may reasonably be assumed that repeated administration during the therapeutic tests would lead to differences in blood concentration even more marked than those recorded here. The agreement between concentration and therapeutic effect suggests that the thiosemicarbazones act directly and not after conversion to metabolites.

It is important to consider the likely reasons for the difference between 8388 and 6057 or 6082, because of their possible bearing on the design of compounds that might attain blood concentrations similar to those of 8388 and have intrinsic activity comparable with that of 6057 or 6082 At present this consideration must necessarily be highly speculative The nature of the blood concentrationtime curves indicates that the main pharmacological difference between the compounds is in rate of elimination from the blood Both 6057 and 6082 appear to be rapidly absorbed, probably more rapidly than 8388 Because most of each compound is degraded in the body it follows that the differences in the rates of elimination are mainly conferred by variation in the rate or manner of degradation This is further supported by the fact that 8388, which is the most persistent compound, is nevertheless excreted in larger amount than the other two

At first sight, the most likely point of chemical attack is the thiosemicarbazone group. If this be so it must be assumed that the group is readily attacked in 6057 and 6082, and only attacked with difficulty in 8388. Some weak indirect evidence in support of this assumption is available, in that Hoggarth (1948) has shown that a methoxy group in the para position of certain compounds related to benzaldehyde thiosemicarbazone renders the thiosemicarbazide

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residue more susceptible to scission However, a spectrophotometric search for metabolites in extracts of urine from rabbits receiving 6057 failed to indicate the presence of free or conjugated anisic acid, which is the most likely end-product of attack on the thiosemicarbazone group Free or conjugated mercaptotriazole and thiodiazole also appeared to be absent Both of these are potential cyclization products of thiosemicarbazones, the thiodiazole can be readily obtained in vitro by mild oxidation The examination of the urine was not exhaustive, and other products may well have been present. In particular, p-hydroxybenzoic acid would probably not have been extracted by the solvents used

A second possibility exists, since the methoxy and hydroxy para substituents of 6057 and 6082 are known to be capable of metabolic modification. whereas the ethylsulphonyl group of 8388 is thought not to be For example, p-methylsulphonylacetanilide is a metabolic end-product of p-methylthioaniline in the mouse, rat, and rabbit (Rose and Spinks, 1948), and sensitive tests failed to detect sulphanilic acid derivatives in the urine of these animals Similar results were obtained with the ethylsulphonyl homologue (Spinks, 1948) therefore probable that no further breakdown of the alkylsulphonyl group occurred These considerations suggest that 6057 may be demethylated in the body to give 6082, 6082 would probably be conjugated with sulphuric acid or glucuronic acid The conjugated derivatives when hydrolysed, would give p-hydroxybenzaldehyde which would almost certainly have escaped detection in the search for metabolites referred to above These views do not directly explain the excretion of only a part of the 8388, but do so if the further assumption is made that the thiosemicarbazone group is slowly, and the para substituents of 6057 and 6082 are rapidly, attacked

A third possibility is that each compound is hydroxylated in the body according to known metabolic reactions, and then conjugated would give a guaracol, and 6082 a catechol derivative, 8388 would probably be hydroxylated in the position meta with respect to the ethylsulphonyl group It can be assumed that this meta hydroxylation would be less facile than the ortho hydroxylation of 6057 or 6082 Some support is available for the last two possibilities in that urine from rabbits receiving 6082 contains a water-soluble compound having an intense absorption spectrum with a sharp maximum at 315 mm. The amount of this metabolite is estimated to be equivalent to about 60 per cent of the amount of 6082 administered, if similar intensities are assumed for the respective absorption

This observation, made after the first draft spectra of this paper had been written, shows, first, that 6082 is not excreted in the faeces to any marked extent, and, second, that the thiosemicarbazone group may well have remained intact, or may have been only partly degraded, say to the corresponding simpler metabolites would be semicarbazone expected to absorb at shorter wavelengths Otherwise there is little evidence in favour of any one of the theoretically possible methods of degradation Indeed it is conceivable that all three could occur However, they all suggest somewhat similar conclusions, 1e, that new antituberculous thiosemicarbazones should be sought among compounds having an interactive meta directing group, or, less probably, an unreactive ortho-para directing group in the para position. It is possible that the requirements for high intrinsic activity and high blood concentrations may be incompatible, numerous examples of weakly active compounds belonging to one or other of these two suggested types (Hoggarth et al., 1949) favour this view It is hoped that a conclusion may be reached, first by studying the metabolism of the three compounds mentioned here, second by examining the blood concentrations of other compounds. A start has been made by determining the concentrations attained by p-acetamidobenzaldehyde thiosemicarbazone, which has been tested in man by Domagk's clinical collaborators (Moncorps and Kalkoff, 1947, Kuhlmann, 1948) So far it appears that this compound gives concentrations in mice similar to those of 6057 and 6082, and that it lacks the favourable persistence of 8388

SUMMARY

Spectrophotometric methods of estimating the thiosemicarbazones (6057, 6082, and 8388 respectively) of p-anisaldehyde, p-hydroxybenzaldehyde and p-ethylsulphonylbenzaldehyde have been devised

6057 and 6082 are rapidly eliminated and attain only low blood or plasma concentrations in mouse and rabbit 8388 is highly persistent and attains high blood concentrations. Only traces of 6057 and 6082 appear in rabbit urine. They are probably almost completely metabolized by the rabbit 8388 is excreted unchanged in larger, but not theoretical, amounts

The bearing of these results on the antituberculous activity of this group of compounds has been discussed

The author is indebted to Dr E Hoggarth for helpful discussion, and to Mrs A Horton for skilled technical assistance

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Note added in proof

The thiosemicarbazones were found to be photolabile during this work, but no evidence of photodecomposition was obtained during the estimations, which were carried out in winter. Repetition of some of them recently has shown that in summer decomposition by diffused daylight can occur, and estimations are now carried out in a darkroom, under diffused tungsten illumination. Readings of the standards indicate whether or not decomposition has occurred

THE EFFECT OF DITHIOLS ON SURVIVAL TIME IN RATS AND MICE POISONED WITH ORGANIC ARSENICALS

BY

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(Received May 19 1949)

The usual method for observing the anti-arsenical activity of dithiols has been to inject two groups of animals with a lethal dose of the arsenical, to treat one group with a dithiol and to compare the mortalities in the two groups (Stocken and Thompson, 1946, et al) As Box and Cullumbine (1947) have pointed out, the use of a quantal response (percentage mortality) for quantitative studies of this sort has considerable disadvantages the dose range in which there is a suitable mortality is small, large numbers of animals must be used to give accurate estimates of mortality, the accuracy of the estimates depends on the observed mortalities and must be weighted accordingly, and it is difficult or impossible to introduce a number of factors into the experiment and to detect interactions between them Box and Cullumbine suggested that similarly useful information might be obtained much more efficiently by using the times of survival after a lethal dose of poison instead of the mortalities Weatherall (1945, 1949) observed that animals poisoned with oxophenarsine and treated with doses of dithiols insufficient to save life lived longer than control animals given no dithiol, and showed that in the controls the log survival time was roughly linearly related to the log dose of oxophenarsine It was therefore decided to investigate the relation between dose of arsenical and survival time more fully, and to study the effect of dithiols on this relation, in order to see whether a more satisfactory method of measuring anti-arsenical activity could be so obtained

MATERIALS AND METHODS

Solutions of phenylarsenoxide were prepared by dissolving crystalline phenylarsenoxide (m p 159–161°C) in 3N sodium hydroxide, neutralizing with 6N hydrochloric acid as nearly as possible without precipitation, and diluting with water until isotonic and then with 0.9 per cent (w/v) sodium chloride solution. Solutions of oxophenarsine (mapharside) were prepared in distilled water from pure material kindly provided by Messrs Parke Davis and Co. The dithiols used are described in

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Table I The preparation and chemical properties have been or will be described elsewhere (Evans and Owen, 1949, Evans, Fraser, and Owen, 1949) Solutions of 1 2-dimercaptopentane-3 4 5-triol, 1 2-dimercaptohexane-3 4 5 6-tetrol, dimercaprol glucoside (BAL-Intrav) and 3(2' 3'-dimercaptopropyl)-mannitol were prepared by dissolving their barium salts in water, adjusting the pH to 6 with 10N sulphuric acid, removing any remaining barium ions with saturated sodium sulphate, and removing the barium sulphate by centrifuging for 15 min at 2,500 r p m The complete removal of barium ions was checked by adding a further trace of saturated sodium sulphate and observing the absence of a precipitate The concentrations of these solutions were estimated at the time of experiments as follows sufficient hydrochloric acid was added to make the solutions of normal acidity, and aliquots were then titrated at 0° C against N/10 or N/50 iodine in potassium iodide with a few drops of 1 per cent (w/v) starch solution as indicator until a blue colour persisted for 30 sec 2 3-Dimercapto propionic acid was dissolved in a minimal amount of 3N sodium hydroxide before dilution with 09 per cent sodium chloride Solutions of other dithiols in 0 9 per cent sodium chloride or in olive oil were diluted so that the volume of each injection was 5 0 ml/kg. All doses have been expressed in microgramme-molecules (μM) per kg body weight, in order to facilitate the comparison of chemically equivalent quantities of different substances, and in order to avoid doses numerically smaller than unity and therefore having negative logarithms

Young female albino rats weighing 55 9±11 9 (S.D.) grammes and adult male mice weighing 20 2 ± 2 9 (S D) Experiments were done in a grammes were used thermostatic room, the temperature varied between 21° C and 25° C on different days, but it was checked half-hourly during experiments and rarely varied by more than ±05° C during each experiment withheld from the animals on the evening before an experiment, and on the following morning each animal was put in a numbered glass jar with a wire mesh top They were then selected for different treatments by use of a table of random numbers (Fisher and Yates, 1943, Each animal was injected intramuscularly with an arsenical in one limb and immediately afterwards with The time of a dithiol or saline in the opposite limb injection was recorded For practical convenience the animals in a group receiving the same treatment were

TABLE

Reference	McDonald (1948)	Stocken and	Fitzhugh et al	Weatherall (1949) Weatherall (1949)		, , , , ,		64	**	Fitzhugh et al	(1946) Weatherall (1949)	Chenoweth et al	(1946) Weatherall (1949)	•
Route of admini-stration	Intramuscu-	.,	•	2 2	•	•	•	•	:	•	66	Intravenous	Intramuscu-	
Species	Rat	î	:	Mouse ",	•			:	•	Rat	Mouse	Cat	Mouse	
LD50 µM/kg	1,220	910	850	(1,000 1,000	1,700–2,500	1,700	3,800-4,700	5,000-8,000	3,200	009	006	ca 700	1,600	
Solvent used		10°14 /00 0	0 9% 14aCl	•	:	•	•	Olive oil	0 9% NaCi	NaOH and	0 9% NaCi	•	Olive oil	
Mol		2	+ 71	154	184	214	286	342	288	138	148	150	250	`
Formula			CH ₂ SH CHSH CH ₂ OH	СН ₂ SН СНSӉ СНОН СН ₂ ОН	CH ₂ SH CHSH (CHOH) ₂ CH ₂ OH	СН ₃ SH СНSH (СНОН) ₃ СН ₃ ОН	CH ₃ SH CHSH CH ₂ OC ₆ H ₁₁ O ₆	CH ₂ SH CHSH CH ₃ OC ₆ H ₇ O(OCH ₃) ₄	CH ₂ SH CHSH CH ₂ OĆ		CH ₂ SH CH CH ₂ CH ₂ CO	CH ₂ SH (CH ₂),CH ₂ SH	CH ₂ (S CO CH ₃)CH(S CO CH ₃)CH ₂ O CO CH ₃	,
Substance		•	2 3-dimercaptopropanol (Dimercaprol BAL)	2-dimercaptobutane-	3 4-diol (1 z-dithio-threitol) 1 2-dimercaptopentane-	3 4 5-triol 1 2-dimercaptohexane-	Dimercaprol glucoside	2 3 4 6-tetramethyl-2' 3'-dimercaptopropyl	glucoside 3(2' 3'-dimercapto-	propyl) mannitol 1 2-dimercaptopropionic	acia 8-mercapto-y-valero- thiolactone	1 6-hexanedithiol	3-acetoxy-1 2-bisacetyl- thiopropane (triacetyl dimercaprol)	(10.14)

injected consecutively, and the groups in which the largest and smallest survival times were expected were injected first and last respectively As is indicated later, this concession to practical convenience was perhaps undestrable The animals were observed about every two minutes for the first few hours and later at longer intervals for forty-eight hours. An animal was deemed dead when it made no respiratory movement while watched for thirty seconds. The time of death was taken as the mean of the time when the animal was last observed alive and the time when it was first observed dead When the logarithmic or reciprocal transformation of the survival times was used, the mean of the logarithms or reciprocals of the times last seen alive and first seen dead was taken. In order to avoid confusion with means

derived from groups of animals, the values for individual animals obtained as just described are referred to hereafter as the survival time, log survival time, and reciprocal survival time, and the word mean is used only in connexion with groups of animals. Any animal which survived longer than forty-eight hours from the time of injection was killed and not counted in the estimation of the mean survival time of its group, except when the reciprocal transformation was used. In fact, only 6 out of 678 animals survived the experimental period. Food and water were withheld during the first ten hours after injection and were then provided ad libitum, though the animals seldom showed any desire to eat or drink.

The statistical procedures and symbols used follow the practice of Fisher (1944), unless it is otherwise indicated

 ${\sf TABLE\ II}$ The effect of dimercaprol on the mean log survival time of phenylarsenoxide poisoned rats

		ose of	Log survival tir	Mean increment			
Exp	dune	rcaprol		Dose of phe	nylarsenoxide		in log survival
p	μΜ/ Log kg μΜ/kg		μM/kg 80 Log μM/kg 1 90	113 2 05	160 2 20	226 2 35	Log hours
A B C D	Nil		+0 063±0 0436 +0 120±0 0738 +0 094±0 0974 +0 068±0 0681	+0 003 ±0 0824 -0 229 ±0 0855 +0 026 ±0 1713 -0 262 ±0 0504	-0 047±0 0515 -0 380±0 1358 -0 200±0 0725 -0 477±0 0661	-0 339±0 0593 -0 606±0 0305 -0 519±0 0691 -0 698±0 0391	
\overline{D}	16	1 20	+0 035±0 0336	-0.199 ± 0.0362	-0 437±0 0196	-0.690 ± 0.0293	0 020±0 3555
D	32	1 50	+0 015±0 0148	-0.081 ± 0.0412	-0 394±0 0418	-0.664 ± 0.0642	0 061±0 0372
D	46	1 66	+0 137,±0 0570	-0 240±0 2132	+0 048±0 1114	-0.680 ± 0.0380	0 159±0 0687
C	80	1 90	+0 538±0 0940	+0 295±0 0524	+0 229±0 0497	-0.225 ± 0.0735	0 359±0 0653
A B C	113	2 05	+0 943±0 1292 +0 676±0 1228 +0 618±0 0736	 +0 506±0 0490	+0 447±0 0756	- +0 001±0 0533	 0 544±0 0638
A B C D	160	2 20	+1 047±0 0696 +1 104±0 0528 +0 964±0 0571 +1 027±0 1626	+0 832±0 0799 +0 697±0 0481 +0 608±0 0351	 +0 560±0 0788 +0 410±0 0681	 +0 135±0 0597 +0 029±0 0918	0 907±0 0499 0 955±0 0472 0 717±0 0628 0 858±0 0645
A B C	226	2 35	+1 114±0 0340*	+0 914±0 0247 +1 171±0 1091 +0 728±0 0862	+0 742±0 0542 +0 513±0 0294 +0 727±0 0474	+0 317±0 0704 +0 327±0 0496 +0 293±0 0366	0 852±0 0391 1 072±0 0591 0 814±0 0648
A B C	320	2 50	 	+1 015±0 0468 +1 378±0 0013*	+1 015±0 1156 +0 770±0 0829 +0 977±0 0343	+0 473 ±0 0222 +0 571 ±0 0614 +0 494 ±0 0347	0 962±0 0492 1 311±0 0567 1 095±0 0394
A B C	452	2 65	<u>-</u>		+1 060±0 0529 +1 081±0 1298 —	+0 889±0 0463 +0 880±0 0822 +0 654±0 0959	1 168±0 0372 1 474±0 0733 —

One survivor in group † Calculated as described in text

RESULTS

In general it was found both for oxophe arsine and for phenylarsenoxide that the survival time became shorter as the dose increased, and that for a given dose of poison the survival time of animals treated with dimercaprol became longer as the dose of dimercaprol was increased. The relations were studied first with oxophenarsine, but, as clearer results were obtained with phenylarsenoxide, the latter will be presented first and the data for oxophenarsine thereafter summarized briefly, mainly to indicate the differences observed

I Survival time of rats poisoned with phenylarsenoxide with or without treatment with dimercaprol—No information has been found about the LD50 of phenylarsenoxide in rats, but it is reported to be about 12 μ M/kg intraperitoneally in mice (Eagle, Doak, Hogan, and Steinman, 1940) In the present experiments intramuscular doses of 36 μ M/kg and over killed all rats not treated with dithiols, and doses of 80 μ M/kg and over were nearly always fatal when the amount of dithiol did not exceed twice the equivalent amount of phenylarsenoxide As survival was not desired, doses of the latter order were used, with the results shown in Table II and Fig 1

Sets of sixteen rats were divided into four groups of four, and the groups were injected with 80, 113, 160, and 226 μM/kg respectively of phenylarsen-Some sets received a dose of dimercaprol which was constant for the set, and one set, the controls, were treated with 09 per cent sodium choride solution Particularly in early experiments the dimercaprol-treated sets were sometimes incomplete, either because groups in which survivors were expected were omitted or because the ranges being explored required more rats than were available or manageable to fill all the groups included in the range When the mean survival times of the groups in each set were plotted against the dose or the log dose of phenylarsenoxide the points lay on a curve and the variance increased as the mean survival time increased Two transformations of the data were therefore examined When the means of the reciprocals of the survival times (cf Box and Cullumbine, 1947) were plotted against the dose or the log dose of phenylarsenoxide, the points again lay on a curve and the variance still increased as the means increased But when the means of the logarithms of the survival times were plotted against the log doses of phenylarsenoxide, the points lay roughly on a straight line and the variances showed only slight positive correlation with the means Moreover, the lines for sets which received

a constant dose of dithiol were approximately parallel to those for the set which received no dithiol in the same experiment, and there was no obvious progressive change of slope with increasing doses of dimercaprol In order to assess the significance of the departures from linearity, analyses of variance were performed on those parts of the data which were symmetrically arranged (Table III) results of these analyses were not altogether satis-The significance of the regression of log survival time on log dose of phenylarsenoxide and the significance of the effect of dimercaprol, except in the smallest doses (experiment D), were indeed beyond question But in experiments A and C the mean squared deviations from linearity were very significantly greater than the mean square attributable to random fluctuation, and experiments B and D showed a less marked but similar tendency experiment B there was also a greater departure from parallelism between the lines in the presence and absence of dimercaprol than would be expected by chance

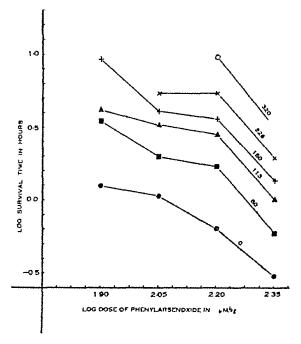


Fig 1—The log survival time of rats poisoned with phenylarsenoxide and treated with dimercaprol (experiment C) Ordinates log survival time in Abscissae log dose of phenylarsenoxide, hours Rats treated with 0.9 per in μM/kg cent sodium chloride Rats treated with dimercaprol, 80 µM/kg —▲ Rats treated with dimercaprol, 113 μM/kg ---+ Rats treated with dimercaprol, 160 µM/kg dimercaprol, 226 µM/kg Rats treated with -O Rats treated with dimercaprol, 320 $\mu M/kg$ All points indicate the mean for groups of four rats

TABLE III

ANALYSIS OF VARIANCE OF THE LOG SURVIVAL TIMES OF RATS POISONED WITH PHENYLARSENOXIDE AND TREATED WITH DITHIOLS

The data analysed here are summarized in Tables II and VI The values selected are, in experiments A and B, phenylarsenoxide 113, 160, and 226 μ M/kg, dimercaprol 0, 226, and 320 μ M/kg, in experiments C, D, E, and F, phenylarsenoxide 80, 113, 160, and 226 μ M/kg and all doses of dithiols for which information was available at the same four doses

The figures in parentheses are the number of degrees of freedom with which the means are estimated Squares for linear regression and (except in experiment D) for treatment with dithiols are all significantly (P < 0.001) greater than the mean square for random fluctuation. Other mean squares are printed in italics when they exceed the mean square for random fluctuation by a ratio greater than the 5 per cent point of e^{3z} and in bold type when the ratio is greater than the 1 per cent point

,			Mean	squares		
Variance due to	Exp	periments w	Experiments with other dithiols			
	A	В	\boldsymbol{C}	D	E	F
Linear regression on log dose of phenylarsenoxide Deviations from linear regression Treatment with dithiols Differences of linear regression Other interaction Random fluctuation	1 462(1) 0 239(1) 3 148(2) 0 036(2) 0 019(2) 0 017(27)	2 742(1) 0 143(1) 5 855(2) 0 135(2) 0 061(2) 0 024(27)	3 929(1) 0 159(2) 1 505(3) 0 016(3) 0 017(6) 0 025(48)	4 346(1) 0 078(2) 0 076(3) 0 004(3) 0 099(6) 0 022(48)	6 329(1) 0 059(2) 1 593(4) 0 102(4) 0 023(8) 0 034(60)	8 534(1) 0 037(2) 2 896(4) 0 199(4) 0 025(8) 0 034(60)

If a sufficiently wide range of doses is examined, the regression of log survival time on log dose of phenylarsenoxide will not be linear At the lower end of the scale survivals will occur and the log survival time will be practically infinite, and at the upper end a limit is likely to occur depending on the time necessary for the transport of phenylarsenoxide from the site of injection to the site of action and possibly for the accumulation there of toxic substances whose metabolism has been inhibited the departures from linearity showed a consistent tendency towards curvature convex towards the zero ordinate, it would not be surprising But this is not the case—the observed irregularities appear to be distributed more or less fortuitously

There appears to be a more plausible explanation of the irregularities As was indicated above, animals in a group receiving the same treatment were injected consecutively, for reasons of practical The estimate of the random fluctuaconvenience tion, or error, of the experiment is based on the deviations occurring within the individual groups, and therefore does not include any measure of the variation in sensitivity of the animals occurring during the two or three hours in which the injections were performed If there is any reason why such variation might be appreciable, the estimate of error cannot be regarded as satisfactory and tests of significance based on it will tend to underestimate the probability of observed discrepancies being due to chance In fact there is present at least one

detectable factor which may account for a greater variation between groups treated at different times than is observed within the groups. It was found that the slopes of the regression lines relating log survival time to log dose (in rats treated only with phenylarsenoxide) did not vary greatly from day to But the position of the line varied, and it appeared that this was due largely to the temperature of the room, and possibly to the weight of the rats A multiple regression relating log survival time in hours (Y) to log dose of phenylarsenoxide in μ M/kg (d_P), body weight in grammes (ν) and temperature of the room in °C (t) was therefore The log survival time was calculated for 129 rats found to be expressed by

 $Y = 546 - 157d_P - 0002w - 0098t$ The regression for body weight was not significant (02>P>01), but those for dose and for temperature were highly significant (P < 0 001 in each case) It therefore appeared that apart from the influence of dose, changes of temperature of as little as half a degree altered the log survival time by approximately 0 05 log hours, or 12 per cent The temperature of the room in which the experiments were performed was not controlled more accurately than this, and the variation due to changes of temperature within these limits is clearly important. It therefore appears legitimate to regard the estimate of random fluctuation as too small and the significance of departures from linearity and parallelism as consequently overestimated

No very good substitute is available, and probably the least unsatisfactory estimate of overall variation would be given by the mean of the sums of squares attributable to deviations from linear regression, to differences of linear regression, and to other inter-Even if the largest mean square among these is taken as a basis of comparison, the regression itself and the effect of dimercaprol in sufficient doses are still most significant. Preferable estimates of the error of certain important comparisons are suggested below

If the lines are parallel, a given dose of dimercaprol produces a constant increment in log survival time irrespective of the dose of phenylarsenoxide On the assumption that apparent differences in slope were not appreciably more than could be attributed to chance, this increment was estimated by deducting the mean log survival time of a set of rats treated with various doses of phenylarsenoxide from the mean log survival time of a comparable set treated with the same doses of phenylarsenoxide and a constant dose of dimercaprol equivalent to estimating the mean of the increments produced by dimercaprol at each dose of phenylarsenoxide) If Z symbolizes the mean increment in log survival time, y_1, y_2, y_m , the log survival times of individual animals treated with doses 1, 2, m, of poison, and z_1, z_2, z_m , the log survival times of individual animals treated with the same doses of poison and a constant dose of antidote, and there are n animals in each group, then

$$Z = \frac{S(z_1) - S(v_1) + S(z_2) - S(v_2) + S(z_m) - S(v_m)}{mn}$$

As the mean square for error given by the analysis of variance is likely to be too small, the variance of Z has been estimated from the deviations within the 2m groups concerned If v_z is the variance of Z, then

$$V_{z} = \frac{S(z_{1} - \bar{z}_{1})^{2} + S(y_{1} - \bar{y}_{1})^{2} + S(z_{m} - \bar{z}_{m})^{2} + S(y_{m} - \bar{y}_{m})^{2}}{2m(n - 1)} \left(\frac{1}{mn} + \frac{1}{mn}\right)$$

Values for Z and $\sqrt{V_z}$ are given in the last column of Table II

The smallest doses of dimercaprol used had no significant effect on the survival time of phenylarsenoxide poisoned rats, and it appeared that about 40 µM/kg was the threshold dose for this effect, 46 µM/kg produced an appreciable increase in survival time, but this was not significant with the size of group used With larger doses the relation between the mean increment in log survival time and the log dose of dimercaprol was practically linear, even when points obtained on different days were considered together (Fig 2) A straight line was therefore fitted to all points where the log survival time was significantly increased (i.e., those

obtained with doses of dimercaprol of 80 μ M/kg and over), as shown in Fig 2 If Z is the mean increment in log survival time for a dose d_{λ} of dimercaprol, this line has the formula

$$Z = 1 \ 18d_A - 1 \ 81$$

 $Z = 1 \ 18d_A - 1 \ 81$ when Z is measured in log hours and d_A in log $\mu M/kg$

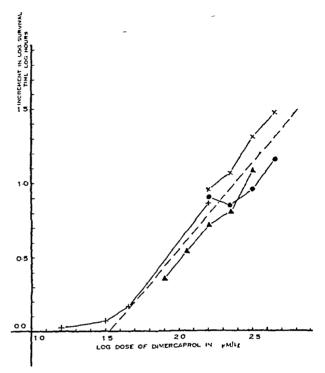


Fig 2 —The increment in log survival time produced by dimercaprol in rats poisoned with phenylarsenoxide mean increment in log survival time Ordinates log dose of dimercaprol in $\mu M/kg$ Abscissae • Experiment $A \times \longrightarrow \times$ Experiment $B - \longrightarrow \times$ Experiment $C + \longrightarrow \times$ Experiment D The line of best fit (---) has been calculated from the equation $Z = 1.18d_A - 1.81$

Examination of the data suggested that within the small range used in the present experiments, environmental temperature had no striking effect on the increment in survival time produced by dimercaprol. and as the data did not give systematic information on this point it was not pursued further

A way of expressing these findings consists in plotting the dose of phenylarsenoxide against the dose of dimercaprol and drawing lines connecting points where the mean survival time is the same Such lines may be called isochrons. In order to prepare these lines, the equations given above for the log survival time in the absence of dimercaprol and for the increment produced by dimercaprol have been used. In the first equation, the mean values for

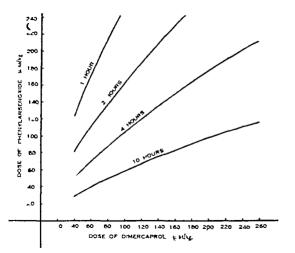


FIG 3—Isochronic lines for rats poisoned with phenylarsenoxide and treated with dimercaprol. Ordinates dose of phenylarsenoxide in $\mu M/kg$. Abscissae dose of dimercaprol in $\mu M/kg$. The values for the points have been calculated as described in the text. The lines, from top left to bottom right, connect points for survival times of 1, 2, 4, and 10 hours respectively.

w (56 g) and t (22°) used in calculating the regression have been substituted, giving the relation between log dose and log survival time as

$$Y = 32 - 157d_P$$

Isochrons obtained by substitution for d_P and d_A are shown in Fig 3. It will be seen that, within the present experimental range the isochronic lines are surprisingly nearly straight, even when the doses and not the log doses are plotted against each other. From these lines, or directly from the regression equations, the amount of phenylarsenoxide neutralized by a given dose of dimercaprol can be calculated. This quantity is not constant. Values for it derived from the one hour, four hour and ten hour isochrons at selected dimercaprol dose levels are shown in Table VII. Their significance is discussed later.

II Survival time of rats and mice poisoned with oxophenarsine, with or without treatment with dimercaprol—The LD50 of oxophenarsine given by intramuscular injection is reported to be in rats between 85 and 93 μ M/kg (Gruhzit, 1935) and in mice about 150 μ M/kg (Weatherall, 1949) Data on the relation of dose to survival time are presented in Tables IV and V. In both rats and mice the transformation log dose/log survival time gave an approximately linear relation with insignificant positive correlation (r = +0.51, 0.1>P>0.05) between the mean and the variance, and with a slope

varying between 1.7 ± 0.33 and 2.3 ± 0.43 on different days Weatherall (1949), with much less accurate estimates of the survival time and with doses which only just caused 100 per cent mortality found a slope of 2.76 in mice. This confirms the expectation that the line is actually convex downwards and can be regarded as approximately straight only over a limited range. The experiments with dimercaprol were designed at a time when it was expected that the dose of dithiol necessary to produce a constant increment in log survival time would be proportional to the dose of poison used quently data are not available for the effect of the same absolute dose of dimercaprol with different doses of oxophenarsine, and lines comparable to those described above for phenylarsenoxide cannot be fitted The increments in log survival time produced by different doses of dimercaprol were not inconsistent with the hypothesis of a linear relation between the mean increment in log survival time

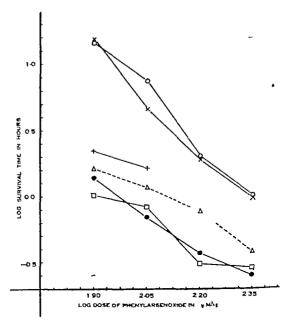


Fig 4—The log survival time of-rats poisoned with phenylarsenoxide and treated with various dithiols log survival time in (experiment F) Ordinates Abscissae log dose of phenylarsenoxide in hours μM/kg •----• Rats treated with 0.9 per cent sodium chloride o----o Rats treated with dimer caprol, 160 μ M/kg \times —— \times Rats treated with 1 2-dimercaptobutane-3 4-diol +--treated with 1 2-dimercaptopentane-3 4 5 triol —□ Rats treated with 1 2-dimercaptohexane 3 4 5 6-tetrol Δ----- Rats treated with δ-dimercapto-γ-valerothiolactone All points indicate the mean for groups of four rats

TABLE IV THE EFFECT OF DIMERCAPROL ON THE LOG SURVIVAL TIME OF OXOPHENARSINE POISONED MICE

		se of	Log sur	vival time (Means	and standard erro	ors for groups of t	en mice)		
Exp	dime	rcaprol	Dose of oxophenarsine						
	μΜ/ Log kg μΜ/kg		μΜ/kg 250 Log μΜ/kg 2 40	320 2 50	400 2 60	500 2 70	600 2 78		
a b c d e	Nıl	~~	+0 712±0 0950 — — — —	+0 391 ±0 0472 +0 357 ±0 0442 +0 437 ±0 0371 +0 516 ±0 0256 +0 460 ±0 0337	+0 284±0 0576 +0 457±0 0824	+0 064±0 0199 +0 176±0 0478 +0 029±0 0557	-0 277±0 1039		
c d e	80	1 90		+0 317±0 0390 +0 477±0 0479 +0 466±0 0229	_ _ _	-			
d e	125	2 10		_		+0 382±0 0266 +0 324±0 0219	_		
c d e	160	2 20		+0 593±0 0101 +0 608±0 1152 +0 424±0 0391	~- ~- 	-			
d e	250	2 40		_	<u> </u>	+0 388±0 1223 +0 374±0 0285			
a b e	320	2 50		+0 814±0 1721† +0 650±0 1203* +0 774±0 1297*					
а b	400	2 60		_	+0 639±0 0781 +0 797±0 1772				
a e	500	2 70	_	=	_	+0 458±0 0701 +0 372±0 0504			

TABLE V THE EFFECT OF DIMERCAPROL ON THE LOG SURVIVAL TIME OF OYOPHENARSINE POISONED RATS

Dose of dimercaprol		206 801 17,101 18,000	Log survival time in hours (Means and standard errors for groups of ten rate				
μM/kg	Log μM/kg	μΜ/kg 320 Log μΜ/kg 2 50	400 2 60	500 2 70			
Nil 80 100 125 200 320 400 500	1 90 2 00 2 10 2 30 2 50 2 60 2 70	+0 011±0 0268 +0 190±0 0408 — — +0 589±0 1263 —	-0 072 ±0 0561 +0 028 ±0 0656 +0 149 ±0 0746 +0 404 ±0 1012	-0 282±0 0689 +0 021±0 0546 			

^{*} One survivor in group † Two survivors in group

and the log dose of dimercaprol The slope, however, appeared to be substantially less than with phenylarsenoxide (0.5 for oxophenarsine in mice, 0.6 for oxophenarsine in rats, and 2.2 for phenylarsenoxide in rats) and the threshold dose of dimercaprol was higher (about $80~\mu\text{M/kg}$) Below this dose, at least in mice, actual acceleration of death was observed, although only once (Table IV, exp. c) was this large enough to be significant with the size of group used

IIISurvival time of rats poisoned with phenylarsenoxide and treated with other dithiols -As in the experiments with dimercaprol, sets of sixteen rats were divided into four groups of four and phenylarsenoxide was injected as before One set received also an intramuscular injection of 0.9 per cent sodium chloride solution, one set the largest dose of dimercaprol which was expected not to save any lives (160 μ M/kg), and the remaining sets the same dose of other dithiols (Table VI and Fig 4) dose was in only one case greater than one-quarter of the LD50 of the dithiol (Table I) instances, titration at the end of the experiment indicated that the solutions contained less dithiol than expected In these cases, the dose was calculated from the results of the titration Insufficient material was available to complete the lines for certain dithiols The variance of the symmetrically arranged data was analysed as before (Table III) For no obvious reason, in neither experiments E nor F were the departures from linear regression of log survival time on log dose of phenylarsenoxide significantly greater than the mean square for error, although the initial fault in the experimental design had not been amended This suggests that whatever in the dimercaprol experiments increased the variation between groups, whether temperature changes or some other unsuspected factor, was less actively operative, and due weight must therefore be attached to the differences of linear regression, which are significantly greater than can be accounted for by random fluctuation The lack of parallelism is hardly surprising Different thiols are likely to be absorbed and excreted at different rates They will reach a peak concentration in the blood and tissues at different times, and so, for example, a slowly absorbed substance might show no appreciable activity against 226 μ M/kg of phenylarsenoxide, which kills rats in about ten minutes, and yet be quite active against 80 µM/kg which is lethal in about an hour Indeed, it can only be fortuitous that dimercaprol happened to be equally active, or nearly so, against all the doses of phenylarsenoxide used Once lack of parallelism occurs, any estimate of activity based on the increment in survival time

becomes an arbitrary measurement depending on the doses of phenylarsenoxide chosen obvious way of overcoming this difficulty. As the conditions in these experiments have been standardized, with four fixed doses of phenylarsenoxide and one dose of dithiol as nearly constant as was practicable, and as the lack of parallelism is not very great, the mean increments in log survival time still give an approximate indication of the activity of the thiol under these experimental conditions, and they have been calculated as before Small differences in the mean increments, particularly when there are also differences in slope, are clearly unimportant. but in fact there were considerable differences in the ability of dithiols to increase the survival time The significance of differences has been estimated as before, using the mean squared deviations of the groups concerned as the basis of comparison in a t test No dithiol was more active than dimercaprol. and all except 1 2-dimercaptobutane-3 4-diol were significantly less active Two substances (1 2dimercaptohexane-3:4 5 6-tetrol and 1 6-hexanedithiol) accelerated death, the latter significantly The other substances were intermediate in activity

To facilitate quantitative comparisons, the activities have been estimated as percentages of the activity of dimercaprol, by assessing from the previously determined dose-response curve how much dimercaprol would have been necessary to produce the observed increments in log survival time. The pooled estimate of the slope of the dose response curve (b = 1.18) was used, and the position of the line was fixed by the observed mean increment in log survival time produced by the standard dose of dimercaprol. For example, in experiment E, $160 \mu \text{M/kg}$ of dimercaprol increased the log survival time by $0.827 \log \text{hours}$, and substituting,

$$0.827 = 1.18 \times 2.20 + a$$
,
 $a = -1.769$

whence

and the expected mean increment in log survival time, Z, for any log dose of dimercaprol, d_A , was given by $Z = 1.18d_A - 1.769$

By substituting for Z-the mean increment in log survival time obtained for an unknown dithiol, e g, 0 445 log hours for dimercaprol glucoside, the amount of dimercaprol to which the dose used of unknown dithiol was equivalent could be calculated

Thus
$$0.445 = 1.18d_A - 1.769$$

whence $d_A = 1.875$

As the log dose of dimercaprol glucoside in this experiment was 2 146 μ M/kg, its activity expressed as a ratio to that of dimercaprol was antilog (1 875–2 146) or 53 7 per cent Values calculated similarly for other dithiols are shown in the third column from the right of Table VI

THE EFFECT OF VARIOUS DITHIOLS AND RELATED SUBSTANCES ON THE LOG SURVIVAL TIME OF PHENYLARSENOXIDE POISONED RATS TABLE VI

rol 1 og) 	ne in hours (Means and four rats)	Log survival time in hours (Means and standard errors for groups of four rats)	s for groups of	;	Slope of line	Molar anti-	Molar	Ratio of
1 og //M// kg // kg // 2 20 // 2 15 // 2 20 //		Dose of phenylarsenoxide	lylarsenoxide		Mean increment in log survival	dose of phenyl-	activity as a	as a per centage of	anti- arsenical
	μΜ/kg 80 Log μΜ/kg 190	113 2 05	160 2 20	226 2 35	Log hours	arsenovide to log sur- vival timo	of that of dimercapiol	that of dimer- caprol	activity and toxicity
	-0 010±0 0291 +1 171±0 0626	-0 188±0 0754 +0 583±0 1297	-0 449 ±0 0670 +0 327 ±0 0243	-0 491 ±0 0894 +0 190 ±0 0524	0 827 ±0 0519	-1 15	18	101	18
	+0732±00993	+0 300±0 1115	+0 012±0 0508	-0 302±0 1046	0 445±0 d585	-2 26	54	24	2 25
	+0 511∓0 0695	+0 165±0 1046	+0 083±0 0530	-0 244±0 0479	0 388±0 0499	-1 56	48	. 31	1 53
	+0 232±0 0460	-0 006±0 1237	-0 187 ±0 1713	-0 374±0 1273	0 176 ±0 0716	-133	28	15	1 82
	+0 146±0 0880 +1 161±0 1012	-0 156±0 0548 +0 875±0 0495	-0 434±0 0853 +0 308±0 0354	-0 596±0 0405 +0 010±0 0105	0 848 ±0 0477	-1 67 -2 68	181	18	18
160 2 20	+1 187±0 0568	+0 660±0 0513	+0 277 ±0 0407	-0 008 ±0 0401	0 789 ±0 0424	-2 64	68	901	68 0
124 2 09	+0 342±0 1367	+0 215±0 0471	I	i	0 289 ±0 0629	1	43	47	06 0
162 2 21	+0 007 ± 0 2083	-0 088±0 1337	-0 507 ±0 1279	-0 538 ±0 1087	-0 022 ±0 0826	-136	0	59	0
160 2.20	+0 208 ±0 0500	+0 60 7 ∓0 0904	$-0\ 112\pm 0\ 0651$	-0 421 ±0 1611	0 196±0 0615	81 1-	78	Ξ	0 25 ,
186 2 20	-0 010 ±0 0479 +0 611 ±0 0686	-0 009±0 0107 +0 683±0 0377	-0336 ± 00750 $+0366\pm00260$	11	0 672 ± 0 0418	11	18	18	18
160 2 20	+0 623 ±0 2396	+0 221 ±0 1252	i	1	0 432±0 0793	1	62	62	00 1
160 2 20	+0 174 ±0 0498	+0 185±0 0593	+0 027 ±0 0238	1	0 247 ±0 0349	1	43	191	0 26
160 2 20	-0 190 L0 0473	-0317±00870	-0 459 土0 0482	Ī	-0 204 ±0 0410	ı	Potentiation	1	I

DISCUSSION

Survival time has not been extensively used in order to measure the effects of drugs in biological Bülbring (1937) showed that extracts of adrenal cortex could be assayed by the increase in survival time produced in adrenalectomized drakes Vogt (1943) showed that this was also true for adrenalectomized rats kept at a low environmental temperature Both showed that the untransformed survival times of treated animals were approximately linearly related to the logarithm of the dose of cortical extract, but the data of Bilbring do not exclude the possibility that the log survival time is as, or more nearly, linearly related to the log dose Box and Cullumbine (1947), using mustard gas and phosgene and measuring the dose as the product of the concentration of the gas and the time of exposure, found that the reciprocals of the survival times were normally distributed and linearly related to the dose of gas and that the logarithms of the survival times were not discussed the merits of using survival time in assays of antidotes to poisons which produce death but no other easily measured response Withell (1942) showed that the log survival times of, microorganisms poisoned with a given concent ation of bactericides were normally distributed, but apparently did not attempt to relate the mean survival time of the organisms to the concentration of bactericide

With the two arsenicals and two species used in this work the logarithm of the survival time was within a limited range approximately linearly related to the logarithm of the dose of arsenical, the logarithms of the individual survival times for any one dose of poison were more nearly normally distributed than were the untransformed survival times or the reciprocals of the survival times, and the standard deviations of the individual values were roughly constant and independent of the dose of poison In animals poisoned with phenylarsenoxide the increment in log survival time produced by dimercaprol was linearly related to the logarithm of In view of the widely the dose of dimercaprol differing conditions of these various uses of survival time, it is perhaps not surprising that the distributions differ greatly and are not all amenable to the same transformations Data obtained under other conditions are necessary before generalizations can usefully be attempted

From the data presented, an attempt can be made to deduce how much poison is inactivated by dimercaprol in the body. Table VII shows what doses of phenylarsenoxide and dimercaprol are required to give certain fixed survival times, according to the

regression equations derived from the data. If the survival time of the rats is regarded as depending entirely on the amount of phenylarsenoxide used in the absence of dimercaprol, then the excess necessary to give the same survival time in the presence of dimercaprol can be regarded as the amount neutralized by the dimercaprol Such amounts are shown in column 4 of Table VII, and are expressed in column 5 as a percentage of the amount of dimercaprol given It appears that the efficiency of the dimercaprol, as judged by the amount of phenylarsenoxide neutralized per molecule, increases with the dose of dimercaprol to a maximum at about 160 μ M per kg and then remains steady at a level depending on the amount of phenylarsenoxide available Not much significance need be attached to the values over 100 per cent in the early part of the Table These values are calculated from two regression equations, in both near or beyond the extremities of the observed values, where the sampling errors of the estimates are largest, the assumption of linearity is most doubtful, and the effect of transforming back from a log dose magnifies discrepancies In practice, no instance has been observed where the increment in survival time produced by dimercaprol was larger than could be

TABLE VII

CALCULATED DOSES OF PHENYLARSENOXIDE AND DIMERCAPROL NECESSARY TO PRODUCE CONSTANT SURVIVAL
TIMES

Survival time Hours	Dose of phenylarsen-oxide, µM/kg	Dose of dimer-caprol μ M/kg	Phenylarsen- oxide neut- ralized by dimercaprol, μ M/kg	Phenylarsen- oxide neut- ralized, mols per 100 mols dimercaprol
1 1 1 1 1	108 123 209 (359) (481)	0 40 80 (160) (240)	15 101 (251) (373)	37 5 126 (157) (155)
4 4 4 4 4 4 4	45 51 87 149 200 245 288	0 40 80 160 240 320 400	6° 42 104 155 200 243	15 52 5 65 64 5 62 5 61
10 10 10 10 10	(25) (28) 48 83 111	(0) (40) 80 160 240	(3) 23 63 86	(7 5) 29 39 5 36

Figures in parentheses are outside the range of experimental observations. The calculation of these figures is described in the text

accounted for by the inactivation of the equimolar quantity of phenylarsenoxide. Nevertheless, in these conditions (near optimal amounts of dimercaprol and excess of phenylarsenoxide) the efficiency of the antidote does approach very close to 100 per cent of what is chemically possible. Figures similarly calculated for oxophenarsine at optimal dimercaprol levels are about 90 per cent at 1 hours, 40 per cent at 4 hours, and 20 per cent at 10 hours, so that although the total amounts of arsenic in the body are larger, dimercaprol appears to be somewhat less effective against this poison

Hogan and Eagle (1944) and Chance, Crawford, and Levvy (1945) showed that in rabbits poisoned with phenylarsenoxide or oxophenarsine phenylarsenoxide was excreted much more slowly than oxophenarsine Chance and Levvy (1947) showed that dimercaprol given to phenylarsenoxide poisoned rabbits increased the excretion of arsenic tenfold, and in oxophenarsine poisoned rabbits increased the excretion of arsenic only 25 times Peters and Stocken (1947) showed that the compound formed in vitro between oxophenarsine and dimercaprol '(4-hydroxymethyl-2-(3'-amino-4'-hydroxyphenyl)-1 3-dithia-2-arsacyclopentane) was much more toxic than oxophenarsine, and that treatment with dimercaprol prevented toxic effects of the oxophenarsinedimercaprol compound In unpublished experiments (Weatherall, 1949) it was shown that when phenylarsenoxide and dimercaprol were mixed in equivalent amounts in aqueous solution a white precipitate formed and when a suspension of this precipitate containing 3 × LD50 of phenylarsenoxide was injected intramuscularly animals showed no ill effects

The toxicity of the oxophenarsine-dimercaprol compound and the small increase in the excretion of arsenic after dimercaprol'in oxophenarsine poisoned animals, compared with the apparent harmlessness of the phenylarsenoxide-dimercaprol mixture and the much increased excretion of arsenic after dimercaprol in phenylarsenoxide poisoned animals probably explains why dimercaprol is less efficient in preventing oxophenarsine poisoning than in phenylarsenoxide poisoning

The other dithiols investigated fall into five chemical classes 1 2-dithiol derivatives of polyhydric alcohols, oxygen ethers of dimercaprol, 1 2-dithiols containing a carboxyl group, $\alpha\omega$ -dithiols and acetylated dithiols. The first group are compounds with the general formula CH₂SH CHSH (CHOH)_nCH₂OH. In this series (exp. F, Table VI) a tivity was maximal when n=0, i.e., in dimercaprol, and fell off as n increased, until 1 2-dimercaptohexane-3 4 5 6-tetrol (n=4) had no activity. This decrease accompanies a decrease in

lipoid solubility The second group were dimercaprol ethers of mannitol, glucose, or tetramethylglucose (exp E, Table VI) The former two were moderately active, but the latter showed little 1 2-Dimercaptopropionic acid in the activity third group was about one and half times as active as δ-mercapto-γ-valerothiolactone Possibly the lactone ring in this compound did not break in vivo, so that the substance contained only one active -SH group However, Weatherall (1949) showed that a dose of this compound equivalent to 154 times the LD99 of oxophenarsine completely prevented mice from dying even when given 80 min after the LD99 of oxophenarsine This suggests that the substance does not act in vivo as a monothiol. because other monothiols, cysteine and glutathione, are useless in preventing death in oxophenarsine poisoned animals when given only 5 min after the oxophenarsine (Eagle, Magnuson, and Fleischman, The only $\alpha\omega$ -dithiol used in this work potentiated poisoning by phenylarsenoxide was surprising because Whittaker (1947) showed that 1 6-hexanedithiol reactivated pigeon brain pyruvate oxidase poisoned with lewisite when added ten minutes after the lewisite, Thomson, Savit, and Goldwasser (1947) showed that skin exposed to lewisite was decontaminated by 1 6-hexanedithiol, although less efficiently than by dimercaprol, and Kensler, Abels, and Rhoads (1946) showed that it was effective in the treatment of arsine poisoning, again less so than dimercaprol On the other hand, Weatherall (1949) found that other αω-dithiols (1 4-dithiothreitol and 1 4-dithioerythritol) accelerated death in oxophenarsine poisoned mice and the former caused a large increase in the mortality from sublethal doses of oxophenarsine It would be interesting to know more about the changes in survival time produced by other doses of 1 6hexanedithiol in phenylarsenoxide poisoned rats Possibly larger doses would have increased the survival time Triacetyldimercaprol, the only acetylated dithiol tested, was about as active as dimercaprol glucoside This is consistent with the results of mortality experiments (Weatherall, 1949)

For practical purposes the ratio of therapeutic activity to toxicity is more important than the absolute therapeutic activity per gramme or per gramme-molecule. As an increase in LD50 represents a reduction in toxicity, the efficiency of these substances has been expressed as the ratio of the anti-arsenical activity and the toxicity, both measured with unit activity as that of dimercaprol, as shown in the last column of Table VI. Only the sugar ethers of dimercaprol surpass dimercaprol when considered in this way, and of the sugar ethers the glucoside is substantially the best

No attempt has so far been made to observe the effect of delaying treatment with dithiols on the relation between survival time and the dose of dithiol, nor to extend the method used in these experiments to determine the relation between other poisons and survival time, or the effects of dithiols on poisoning by other substances

A substance which increases the survival time of experimental animals is not necessarily an effective therapeutic agent in either animals or men Weatherall (1949) has shown that all the substances discussed here with the exception of 1 2-dimercaptohexane-3 4 5 6-tetrol, 1 2-dimercaptopropionic acid, and 1 6-hexanedithiol, which he did not test, reduced the mortality in oxophenarsine poisoned mice, and the compounds which he found most efficient in preventing death in oxonhenarsine poisoned mice are those which in the experiments now reported have been found most efficient in prolonging the survival time of phenylarsenoxide poisoned rats. In the present work the efficiency of the dithiol has been measured only when it has been given immediately after the poison. It is likely that the relative efficiency of different dithiols will differ when they are used in the delayed treatment of acute poisoning or in chronic poisoning. It is, however, unlikely that a dithiol which has no activity when given immediately after the poison, at a time when the poison is circulating freely, will have appreciable effect in chronic poisoning when the poison has been fixed by the tissues The present procedure, therefore, provides a test for excluding ineffective substances, and a quantitative method for differentiating between substances with almost equal activity and for studying changes in activity produced by changes in chemical structure

SUMMARY

- 1 Lethal doses of phenylarsenoxide and oxophenarsine have been injected intramuscularly into rats and mice and the survival times have been measured
- 2 Over the range of doses used the log survival time was as a rule linearly related to the log dose and at least with phenylarsenoxide to the temperature of the room also
- 3 Some poisoned animals were immediately injected intramuscularly with dimercaprol Those treated with dimercaprol lived longer than those untreated
- 4 For a given dose of dimercaprol, the relation between the log dose of phenylarsenoxide and the log survival time was linear and the slope was the same as in the absence of dimercaprol

- 5 The mean increment in log survival time produced by different doses of dimercaprol in phenylarsenoxide poisoned rats was linearly related to the log dose of dimercaprol. This relation was not grossly affected by small differences in environmental temperature.
- 6 From a consideration of the two regressions, it appeared that the amount of phenylarsenoxide inactivated by dimercaprol increased to a maximum when the dose of dimercaprol was about $160 \mu M_{\odot}$ kg and was greater with large doses of phenylarsenoxide
- 7 Similar data are presented for oxophenarsine and dimercaprol in rats and mice. The results are less comprehensive, but suggest that in very small doses dimercaprol can accelerate death in oxophenarsine poisoned mice, and that with larger doses of dimercaprol the relationship resembles that to phenylarsenoxide.
- 8 In the presence of dithiols, the relation between the log dose of phenylarsenoxide and the log survival time was linear but not always parallel to the line for phenylarsenoxide alone. The differences were not large and approximate comparisons of the activity of ten dithiols and related substances have been made and are discussed None of the substances was more active than dimercaprol

We are deeply indebted to Professor J H Gaddum and to Mr D J Finney for most helpful discussions and advice, to Dr G A Levvy and Dr I D E Storey for kindly providing crystalline phenylarsenoxide, to Dr J S White, of Parke Davis, Ltd, for gifts of pure oxophenarsine (mapharside), to Dr L N Owen for gifts of 1 2-dimercaptobutane-3 4-diol, 1 2-dimercaptopentane-3 4 5-triol, 1 2-dimercaptohexane-3 4 5 6-tetrol, 2 3 4 6-tetramethyl 2'3'-dimercaptopropyl glucoside, 3(2' 3'-dimercaptopropyl) mannitol, 8-mercapto-y-valerothiolactone and triacetyldimercaprol, to Dr L A Stocken for gifts of 1 2-dimercaptopropionic acid and 1 6-hexanedithiol, to Miss Irene Munro for technical assistance, and to the Medical Research Council for defraying the expenses of this work and for a personal grant to one of us (J A C W)

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DIMERCAPROL AND THE BILIARY EXCRETION OF LEAD IN RABBITS

RY

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Ginsburg and Weatherall (1948) showed that dimercaprol injected intramuscularly in rabbits shortly after lead acetate had been injected intravenously increased the amount of lead in the alimentary canal and particularly in its contents, and decreased the amount in the liver. The concentration of lead in bile obtained from the gall bladder was very variable and did not provide good evidence about the part played by biliary excretion in this redistribution. The present experiments were directed to settling this point.

METHODS

The procedures used in preparing solutions, administering and estimating lead by means of the tracer Ph²¹² (thorium B) have already been described (Ginsburg and Weatherall, 1948)

In order to collect samples of bile, rabbits weighing 2 5-2 9 kg were anaesthetized with intravenous pentobarbitone or ether and cannulae were tied into the trachea, the internal carotid artery and the common bile The cystic duct was tied The arterial blood pressure was recorded, and artificial respiration was applied if necessary Lead acetate (2 07 mg Pb/kg) containing about 200 microcuries of thorium B was then given intravenously and bile was collected for the next four periods of one hour, and their content of lead estimated In some rabbits, dimercaprol (12 5 mg/kg) in 66 per cent (v/v) aqueous propylene glycol (0 25 mg/ kg) was injected intramuscularly two hours after the lead acetate, and into the rest 66 per cent propylene glycol alone This dose is one-quarter of that previously used for the initial injection, and has been reduced because it is doubtful whether rabbits already subjected to anaesthesia and operative trauma would tolerate much larger amounts In one rabbit (No 355) the dose of dimercaprol was 4 mg/kg After 4 hours the rabbits were killed with a large dose of pentobarbitone and certain tissues were taken for the estimation of the lead in them

RESULTS AND DISCUSSION

The results are shown in Table 1 and Fig 1 When dimercaprol was not administered, the highest

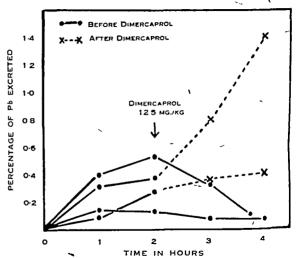


Fig 1 —The effect of dimercaprol on the excretion of lead in bile Abscissa time in hours after the intravenous administration of lead acetate (2 07 mg Pb/kg) Ordinate percentage of administered lead excreted per hour in bile

concentration of lead in the bile occurred in the second hour and later samples contained less. When dimercaprol was injected after the second hour, even in a dose as small as 4 mg/kg, the concentration continued to rise, except in the rabbit anaesthetized with ether. This rabbit was in poor condition and died before the end of the third hour and it is unlikely that the lack of response was due directly to the different anaesthetic. In all these experiments there was a progressive decline in the blood pressure without striking changes referable to the lead acetate or dimercaprol. There was no indication that the increased excretion after dimercaprol was due to circulatory improvement.

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TABLE I

THE EFFECT OF DIMERCAPROL ON THE FATE OF LEAD IN ANAESTHETIZED RABBITS WITH CANNULATED BILE DUCTS, AFTER THE INFRAVENOUS ADMINISTRATION OF LEAD ACETATE (2 07 mg Pb/kg)

					5				118	194							
Rabbit number — Weight and sex —	2 9	323† 2 9 kg đ		353 2 5 kgđ	₹0		355 2 9 kg (O !	(4	317 2 6 kg	*0		345 2 5 kg	₩		356* 2 5 kg	O l
Anaesthetic —						Pent	Pentobarbutone	ttone								Ether	
Dose of dimercaprol mg /kg		ZīZ		ž			4 0			12.5			12.5			12.5	
	Bric % of of mil) dose	μg Pb per sc ml	Brle vol (ml)	of dose	μg Pb per ml	Bile vol	of dose	ug Pb per ml	Bufe vol (ml)	of dose	μg Pb per mí	Bile vol (ml)	of dose	μg Pb per ml	Bule vol (ml)	of dose	μg Pb per ml
Hepatic bile 1st hr ", ", 2nd", ", ", 3rd",	12 0 0 40 9 0 0 54 6 9 0 34	0448	86 7 8 8 8 8	0 15 0 08 0 08	0 78 1 0 0 57	800	0 11 0 22 0 23	0 72 1 4 1 6	81 72 63	0 12 0 24 0 36	0 88 1 9 3 2	386	0 38	4 8 8 1 1 6 1 1 6 1 1 6 1 1 6 1 1 1 1 1 1	36	4900	0 53 1 4 0 85
" " Total " "				0 08	0 61	7	0 38 0 94	8	<u>∞</u> ,		1 9			21		0 24	11
	dosc	μg Pb per gm	dose	!	ug Pb per gm	% dose		ug Pb	% dose	- g	нв Рb per gm	dose		μg. Pb per gm	dose	-	μg Pb per gm
Liver at death Small intestine at death Small intestine contents at	43 2 4	31 2 0	33 0 21		31 0 17	53	1	29 1 4	71 0 90	<u> </u> 	67 0 88	36	1	40	32 0 74	 	25 0 70
deuth ; Blood ;	0 38	11	0 12 5 0		0 70 1 5	0 49		3.5 0.90	99 0		1.5	0 33 5 1		17	0 0		0 02
							-			-			_				

† Died 3 hrs 45 min after injection of lead

* Died 2 hrs 40 min after injection of lead

The average amount of lead in the liver was not reduced by dimercaprol in the rabbits and the amount of lead in the small intestine in one of the control animals was much higher than any previously observed by us. As the bile duct was ligated, this suggests that lead is also excreted directly by at least some part of the alimentary canal. The high value was associated with considerably more handling of the gut than usual in the operative procedure and was perhaps due to consequent hyperaemia. The present results for tissues are very variable and cannot be compared with the findings in normal rabbits.

SUMMARY

Dimercaprol increases the excretion of lead in the bile of anaesthetized rabbits

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pA_x AND COMPETITIVE DRUG ANTAGONISM

BY

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In a recent paper Guarino and Bovet (1949) have studied the use of the measure pA for expressing intensity of drug antagonism pA_x has been defined (Schild, 1947) as the negative logarithm of the molar concentration of an antagonist which reduces the effect of a multiple dose, x, of a stimulant drug to that of a single dose Guarino and Bovet have pointed out that this definition does not specify concentration of active drug, and therefore by implication assumes that pA is independent of the

initial concentration of active drug used In studying the antagonism between acetylcholine and flaxedil* on the frog's rectus abdominis, however, they found that pA was not entirely independent of the initial concentration of acetyl-For instance, choline used an increase in the concentration of acetylcholine of 1 5 log units produced a decrease of pA₂ of 0.53 units Guarino and Bovet then proceeded to evolve a new formula for the

quantitative relations of antagonistic drugs whereby pA would be dependent on the concentration of active drug in competitive antagonism

This interesting paper raises several issues connected with the use of pA and the definition of competitive drug antagonism. I propose to discuss the following points

- 1 Conditions under which pA is independent of concentration of active drug
 - 2 Quantitative relations of antagonistic drugs
 - 3 The practical use of pA

DEPENDENCE OF pA ON HEIGHT OF CONTRACTION
TYPES OF CONCENTRATION-ACTION CURVES

It is well known that concentration-action curves plotted on a logarithmic scale, first in the absence of an antagonist and then in the presence of antagonist, are frequently parallel. This experimental fact forms the basis for Gaddum's formula (1937) for drug antagonism and it is also at the basis of the pA measure, since parallelism on a log abscissa implies that pA is independent of height of contraction (and hence of concentration of active drug). A simple way of finding whether pA is applicable is thus to draw concentration-action curves on a log scale with and without antagonist. This method may also be used to determine pA values.

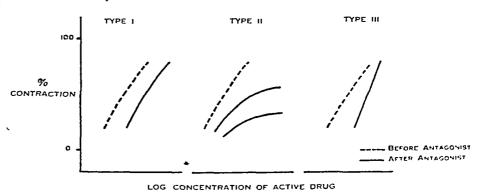


Fig 1 —Three types of concentration-action curves

Concentration-action curves have also been described, however, which are not parallel when plotted on a logarithmic scale, and where pA in consequence is dependent on the height of contraction Amongst these, two types emerge One type (type II of Fig 1) has been described by Zadina (1947) with the following two characteristic properthe concentration-action curves become progressively flatter and their maxima decline as the concentration of antagonistic drug is increased Examples are histamine-harmine on guinea-pig ileum (Zadina, 1947), acetylcholine-magnesium on guinea-pig ileum (Zadina and Kriz, 1948), acetylcholine octyltrimethylammonium on rat's intestine (Clark and Raventos, 1937) Particularly in Zadina's careful experiments it is perfectly clear that not only the slopes but also the maxima of the curves are These results are presumably due to decreased some sort of non-competitive antagonism since the effects of the antagonist cannot be completely

^{*1 2 3-111-\}beta-diethylaminoethoxybenzene trie*kiodide

reversed Such curves might occur, for example, if a constant number of contractile elements (or "receptors") were put out of action by a given concentration of antagonist This would have the effect of decreasing both slopes and maxima

Lastly there are curves of type III (Fig. 1), for example Guarino and Boyet's curves obtained with acetylcholine-flaxedil on the frog's rectus, here the concentration-action curve with antagonist is steeper than without. It was in order to account for these results that Guarino and Bovet suggested a new formula for competitive drug antagonism

OUANTITATIVE RELATIONS OF COMPETITIVE ANTAGONISTS

Gaddum (1937) and Guarino and Bovet (1949) have suggested formulae to express quantitatively the relations of antagonistic drugs competing for receptors The two formulae are incompatible: it can be shown, in fact, that according to one of them pA is independent of concentration of active drug. whilst according to the other it increases as the negative logarithm of concentration of active drug Since the matter is of general interest I shall try, in the following, to point out the differences underlying these two formulations of competitive antagonism

Gaddum's formula is based on the assumption that drug and antagonist compete for free receptors on the cell surface according to a simple mass action law, and that at equilibrium the number of drugreceptor combinations is equal to the number of drug-receptor dissociations and similarly for the antagonist Contraction of the muscle is supposed to be proportional to the average number of receptors occupied by the active drug With these assumptions a formula can be derived which relates percentage contraction y to concentration A of stimulant drug and B of antagonistic drug

$$\frac{y}{100 - y} = K_1 A = \frac{K_1 x A}{K_2 B_x + 1}$$

$$K B_x = x - 1$$
(1)

A similar equation is obtained by applying Langmuir's adsorption isotherm to the case of two gases competing for the same surface (Taylor, 1931)

Guarino and Boyet's formula is derived as follows

Assuming concentration A of a drug to produce effect 1, and concentration 2A to produce a bigger effect, what concentration of antagonist is required to reduce the effect of 2A to that of A? To simplify the argument it is assumed that the antagonist (" false drug") has the same affinity for receptors as the active (" true") drug has The answer is that the concentration of false drug required is equal to that of true drug, namely 2B = 2A, since

in this way the chances of true drug reacting with receptors will be halved and hence its action will be reduced to that of A Generalizing this argument. if the concentration of true drug is increased to xA. then the amount of false drug required to reduce this effect to that of A must be such that the total amount of true and false drug present in the solution is x times the amount of true drug present. This leads to the following general formula

$$K_{\bullet}B_{x} = x(x-1)K_{1}A \qquad (2)$$

where B_x is concentration of antagonist required to reduce the effect of concentration xA of stimulant drug to that of A

In non-competitive antagonism this formula is assumed to reduce to simple proportionality, namely

$$K_2 B_x = x K_1 A \tag{3}$$

This formula, incidentally, was originally used by Clark (1926) to formulate the antagonism acetylcholine-atropine, K_1/K_2 , depending on the effect produced

That the three equations are incompatible may be shown by transforming each to give the antagonist ratio B_{x_2}/B_{x_1} , required to balance an increase of active drug from x_1 -fold to x_2 -fold

From equation (1) $\frac{B_{x_2}}{B_{x_1}} = \frac{x_{2-1}}{x_{1-1}}$

From equation (2)
$$\frac{B_{x_2}}{B_{x_1}} = \frac{x_2}{x_1 - 1}$$
From equation (2)
$$\frac{B_{x_2}}{B_{x_1}} = \frac{x_2(x_2 - 1)}{x_1(x_1 - 1)}$$
From equation (3)

١,

$$\frac{B_{x_2}}{B_{x_1}} = \frac{x_2}{x_1}$$

In this way the applicability of one or the other equation may be tested For example, in order to balance an increase of active drug from 2-fold to 10-fold the antagonistic drug would have to be increased 9 times, 45 times, or 5 times respectively, according to the three equations Unfortunately, this test is not very fruitful, since all sorts of ratios seem to occur, for example, a B_{10}/B_2 ratio of 5 was found for acetylcholine-atropine, 10 for histaminebenadryl (Schild, 1947), and 21 for acetylcholineflaxedil (Guarino and Bovet, 1949)

With regard to pA the following difference exists between equations (1) and (2) From (1), $B_x =$ Therefore B_{τ} is not a function of ASince $pA_x = -\log B_1$, it also is not a function of A Hence whenever equation (1) applies pAx must be independent of concentration of stimulant drug

From (2) B_r is proportional to A Therefore pA is inversely related to concentration of stimulant

drug Thus an increase in the concentration of stimulant drug by one log unit should cause pA to diminish by one unit

In Guarino and Bovet's experiments pA did not decrease to the extent required by their theory, but they regard equation (2) as a limiting case of perfect competitive antagonism which has not yet been shown to occur

Guarino and Bovet's assumptions differ in two respects from the assumptions underlying, eg, equation (1)

- (1) It is assumed that if to a given concentration of true drug a quantity of false drug be added to increase total concentration x-fold, then the chances of true drug reacting with receptors would be reduced to 1/x By equation (1) this would only apply if all receptors were initially occupied Otherwise the addition of antagonist would increase the total number of receptors occupied and the chances of reacting would be reduced to 1/x of the new total and not of the original
- (11) It is assumed that reducing the chances of receptor combination to 1/x by false drug has the same effect as decreasing the concentration of true By equation (1) this would apply drug to 1/xapproximately when a few receptors were occupied, but would not apply when most receptors were This may be illustrated as follows Assume a sufficient concentration of true drug present to produce an almost maximal effect this concentration were halved the effect would be only slightly decreased since most receptors would still be occupied If, however, an equivalent amount of false drug were added each receptor would have an equal chance of being occupied by one or the other and the effect would be halved

In conclusion Guarino and Bovet's assumptions do not appear to be based on mass action competition for receptors in the sense that drug or antagonist occupy a number of receptors for a finite time to the exclusion of each other. Langmuir's treatment is based on the assumption that after condensation of a molecule of the gas owing to adsorption on an "elementary space" a finite interval elapses before it escapes again from the surface. This notion gives a clear physical meaning to the term competition, which is absent in Guarino and Bovet's hypothesis

THE PRACTICAL USE OF pA

Methods of measuring activity of antagonistic drugs may be divided into comparative and non-comparative methods and into methods in which the antagonist is injected first (preventive) and methods in which the active drug is injected first

(curative) pA belongs to the group of non-comparat ve and preventive methods and in this way its field of usefulness is limited For example, it is difficult to use a preventive method quantitatively in the whole animal since this requires producing a constant concentration of antagonist in the blood stream, on the other hand it is often possible to produce a constant effect with the active drug and reverse it by means of an antagonist (e.g., Ing. Dawes, and Wajda, 1945) The main disadvantage of pA is its variability, which is greater than that of an assay relying on a comparison with another antagonistic drug For this reason comparative assays are likely to be preferred for routine work, but for the purposes of a general scale an absolute method and one in which the effect of time and concentration can be accurately assessed is preferable (Schild, 1947)

One way of decreasing variability is to define in great detail the conditions of the experiment. There is little purpose, however, in introducing arbitrary limitations which do not add to our information, unless some important systematic error is thereby avoided. For example, it is probably not necessary to choose a uniform strain of guinea-pigs since pA does not appear to vary systematically from one laboratory to another (cf. results of Reuse (1948) and Schild (1947))

In an antagonist assay on an isolated preparation the following variables may determine the effect experimental preparation, concentration of antagonistic drug, time of contact with antagonistic drug, concentration of active drug, effect produced by active drug alone, composition of Ringer's fluid, temperature, etc. In measurements of pA only the first two or three are defined. By implication the others may be assumed to introduce no important systematic variation.

In practice a compromise must be found between having too many arbitrary limitations and excessive variability For example, in the method of Miller. Becker, and Tainter (1948) the concentration of active drug is fixed This introduces an arbitrary limitation, but it certainly helps to reduce variability In the case of pA neither concentration of active drug nor effect produced by active drug is defined. hence the assumption is that for practical purposes pA is independent of these variables obviously not true when dealing with concentrationaction relations of type II and III above, and I would suggest that in such cases pA be measured when the effect is 50 per cent of the maximal. In this way pA may be used as a practical measure of antagonistic activity even when it is not independent of concentration of active drug

SHMMARY

- 1 Three types of concentration-action curves have been described when the effect of a drug on an isolated tissue is plotted against the logarithm of the dose, in the absence and presence of antagonist Curves of type I remain parallel, those of type II become flatter, and those of type III steeper in the presence of antagonist Type II is often associated with a lowering of the maximum contraction (Zadina) By definition pA is constant only when curves of type I occur
- 2 Guarino and Bovet have recently shown that curves of type III may occur and have suggested a formula which would account for these curves Gaddum had previously suggested a different formula for competitive drug antagonism, which accounts for curves of type I The two formulae are contrasted and the conclusion is reached that Guarino and Bovet's formula implies a type of antagonism which does not involve the notion that drug and antagonist compete for and occupy the same

receptors according to a law of mass action. The available experimental data are insufficient to distinguish between the two views

3 Variability may be diminished by defining the height of contraction at which pA should be determined. With curves of type I it is unnecessary thus to define the effect, but in other cases a 50 per cent contraction may be chosen for determining pA

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SOME PHARMACOLOGICAL PROPERTIES COMMON TO ANTIHISTAMINE COMPOUNDS

BY

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In recent communications (Dutta, 1948, Burn and Dutta, 1948, Dutta, 1949) it has been shown that \beta-dimethylaminoethyl benzhydryl ether hydrochloride ("Be adryl," Parke, Davis and Co, Ltd) possesses some pharmacological properties in common with atropine, pethidine, procaine, and quinidine, it produces a fall of body temperature in mice, abolishes the constrictor action of adrenaline and histamine on the perfused vessels of the rabbit's ear, and relieves histamine-induced bronchoconstriction in the guinea-pig It was also demonstrated 2-(N-phenyl-N-benzylaminomethyl)iminazothat line hydrochloride ("Antistin," Ciba, Ltd, or "Histostab," Boots' Pure Drug Co, Ltd) shares some of these properties, for it, too, antagonizes the action of adrenaline on the rabbit ear vessels and is only slightly less effective than benadryl in alleviating bronchial constriction in guinea-pigs after the intravenous injection of histamine The investigation of these substances has since been continued, and in this paper the actions of β-dimethylaminoethyl benzhydryl ether hydrochloride (which will be called benadryl) and 2-(N-phenyl-N-benzylaminomethyl)ımınazoline hydrochloride (which will be called antistin) on cardiac and skeletal muscle and on ganglonic transmission are described results of an investigation of benadryl on gastric acidity induced by histamine are also presented

Local anaesthetic action

The majority of the common antihistamine substances are local anaesthetics. This has been shown to be true for benadryl (Leavitt and Code, 1947) and antistin (Brack, 1946) in man and in frogs (Reuse, 1948). Graham (1947) referred to the local anaesthetic action of these substances on guinea-pigs but did not give any details of his findings. In the present investigation the strength of these substances was determined in relation to procaine by the intracutaneous weal method in guinea-pigs des-

cribed by Bülbring and Wajda (1945) In guineapigs the sensitiveness of the different areas of the skin on the back varies considerably. In order to reduce this to a minimum, Somers and Edge (1947) suggested the use of a statistical design of Latin squares for the arrangements between the doses and the sites at which the substances are to be injected. This was adopted. Three animals were

TABLE I
EXPERIMENTAL DESIGN, SHOWING THE POSITION OF THE
INJECTIONS ON THE BACK OF THE GUINEA-PIGS

	Areas	Gu:	Guinea-pig		
1st day—low dose	α	P	B	A	
	β	A	P	B	
	γ	B	A	P	
2nd day—middle dose	α	A	P	B	
	β	B	A	P	
	γ	P	B	A	
3rd day—high dose	α	B	A	P	
	β	P	B	A	
	γ	A	P	B	

 $a = front \quad \beta = middle \quad \gamma = back \quad P = process \quad A = antistin$ B = benadryl

used for each comparison. The back of each guineapig was divided into 9 squared areas. Procaine, antistin, and benadryl were injected intradermally in three different concentrations (for each substance) on three consecutive days. All these compounds were given at the same dose level on any one day, and each animal received each substance but at different sites. This is shown in Table I. On the first day of the test guinea-pig 1 received low doses of procaine, antistin, and benadryl in the front, middle, and rear part of the back respectively on the left-hand side. The reaction to 6 pin pricks was

TABLE II

NUMBER OF PRICKS (OUT OF 36) FAILING TO ELICIT A RESPONSE AFTER INTRACUTANEOUS INJECTION OF PROCAINE, ANTISTIN, AND BENADRYL IN THE GUINEA-PIG

Compounds	h	Procaine ydrochlori			Antistin	-		Benadryl	
Concentration in per cent (w/v)	0 1	0 25	10	0 04	0 1	0 25	0 04	0 1	0 25
Pig 1 ,, 2 ,, 3 ,, 4 ,, 5 ,, 6	8 13 4 5 5	7 20 21 18 16 7	30 26 36 22 31 36	6 4 8 3 4 11	7 20 16 9 15 17	29 29 31 5 35 12	5 7 12 3 4 7	8 26 20 18 20 21	31 24 36 15 24 36
Mean	7 5	15	30	6	14	, 23 5	63	19 0	27 6

determined at each site every five minutes. The total number of times a prick failed to produce a response in the course of half an hour, out of a possible 36, indicated the degree of anaesthesia in that particular spot. Next, guinea-pig 2 was injected with benadryl in the front, procaine in the middle and antistin in the rear part on the left squared areas as before and so on. On the second day the injections were given in the middle areas and on the third day on the right-hand side squares. Two complete series of experiments were carried out with 6 guinea-pigs.

There were 54 individual comparisons at different sites on 6 guinea-pigs The results are summarized in Table II Each of the figures in Table II (except at the bottom line) represents the total number of pricks, out of 36, which failed to elicit a response at a given site during the half hour observation. The mean values for the six experiments are given at the base line Variation in the sensitiveness of the different areas of the skin is evident if some of the individual figures are examined For instance, antistin in 0 25 per cent concentration produced less effect than in 0.1 per cent in guinea-pig 4 Similar results were also obtained from guinea-pig 6 higher concentration of benadryl caused a lower degree of anaesthesia than the lower concentration of the drug on guinea-pig 4 These are the extreme examples

When the mean figures indicating the degree of anaesthesia for any one of the given substances (shown in Table II) are plotted as ordinates against its logarithmic concentrations as abscissae, a linear relationship is obtained This is illustrated in Fig 1. The slopes of the lines for procaine, antistin, and benadryl are approximately parallel within the doses.

mentioned above This parallelism did not exist when higher concentrations of antistin or lower doses of benadryl were used. At dose levels where all the three substances prevented 50 per cent of the sk n reactions, benadryl was 3 2 times and antistin 2 3 times as potent as procaine.

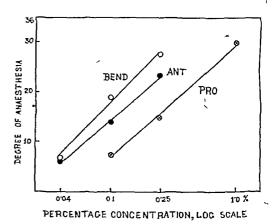


FIG 1—Intracutaneous weal in guinea-pigs The graph shows the relation between the concentration of the local anaesthetic (abscissae) and the number of times there was no response to a prick, total 36 pricks in 30 min (ordinates) Each point represents the mean of six observations BEND = benadryl, ANT = antistin, PRO = procaine hydrochloride

Antagonism to acetylcholine on cardiac muscle

It is known that both antistin and benadryl antagonize certain actions of acetylcholine, and consequently whether they would also antagonize acetylcholine on the isolated rabbit's auricles beating in oxygenated Ringer-Locke's solution at 29° C was investigated Both these substances reduced

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Tel: Fig 2—Isolated rabbit auricles Ringer-Locke's solution at 29° C, 50 ml bath Upper record Depression caused by 10 µg acetylcholine for 2 min (at the end of which the solution was changed, W) at 6-min intervals second dose of acetylcholine was preceded 1 min before by 500 μ g antistin hibition due to acetylcholine was reduced Lower record Depression caused by 50 µg acetylcholine for 2 min (at the end of which the solution was changed, W) at 5-min intervals, 500 µg benadryl was given 40 sec ANTISTIN before both the second and third doses of acetylcholine The effect of acetylcholine was greatly diminished in the presence of benadryl

BEND 50, ACH

50_ACH

BEND 50 ACH

the depression caused by acetylcholine on the auricular contractions. Repeated washings were required before the inhibition produced by acetylcholine was fully recovered. The experiments are illustrated in Fig. 2

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Effect on the refractory period of the cardiac muscle

Dawes (1946) stated that the quinidine-like action of a substance is associated with its ability to oppose the action of acetylcholine Benadryl and antistin

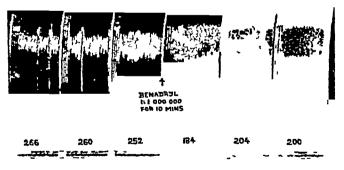


FIG 3—Isolated rabbit auricles stimulated by break induction shocks Ringer-Locke's solution at 29°C, bath 100 ml The auricles fail to follow up to 260 stimuli per min, but follow 252 per min After 10 min exposure to 1 1,000,000 benadryl at the arrow (the drum was stopped for 9 min) the auricles follow up to 200 but fail to follow 204 stimuli per min

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were, therefore, tested on the isolated rabbit's auricles stimulated electrically in order to determine whether their power to antagonize the action of acetylcholine was associated with a quinidine-like The method followed was that of Dawes The isolated rabbit's auricles were suspended in a 100 ml bath of oxygenated Ringer-Locke's solution (containing double the usual amount of glucose) at 29° C The effect of benadryl is illustrated in Fig. 3 Before addition of benadryl to the bath the auricles were able to follow 252 stimuli per min but not 260 per min or more After 100 µg benadryl had been added to the bath the auricles first followed stimulation at 184 per min but not at 204 per min and finally followed 200 per min Benadryl, therefore, in a concentration 1 in 1,000,000 reduced the maximal rate by 20 6 per cent. The relation between the percentage decrease in the maximal rate at which the auricles could follow electrical stimen and the logarithm of concentrations is linear Similar results were obtained with antistin. The results of all the experiments are summarized in Table III antistin, the effect of benadryl on the auricles is more persistent, therefore, when its action was compared with that of quinidine, not more than two doses of each of the substances were used on any individual preparation. Benadryl and antistin are about twice as active as quinidine at the level at which the three substances reduce the maximal rate by 15 per cent

TABLE III

COMPARISON OF BENADRYL, ANTISTIN, AND QUINIDINE ON
THE "DRIVEN" RABBIT AURICLE

Exp No	Substances	Conc	Percentage reduc- tion in the maxi- mal rate at which the auricles followed
4	Benadryl ,, ,	$\begin{array}{c} 10^{-6} \\ 2 \times 10^{-6} \\ 4 \times 10^{-6} \\ 8 \times 10^{-6} \end{array}$	10 5 16 6 23 0 27 5
5	Benadryl Quinidine HCl	$ \begin{array}{c} 10^{-6} \\ 2 \times 10^{-6} \\ 10^{-6} \\ 2 \times 10^{-6} \end{array} $	20 6 39 3 8 2 29 0
6	Benadryl	4×10^{-6} 8×10^{-4} 1.6×10^{-6}	10 0 14 1 23 0
7	Benadryl Quinidine HCl	$ \begin{array}{c} 10^{-6} \\ 2 \times 10^{-6} \\ 10^{-6} \\ 2 \times 10^{-6} \end{array} $	13 1 20.2 8 4 14 0
8	Quinidine HCl Benadryl	$ \begin{array}{c c} 10^{-6} \\ 2 \times 10^{-6} \\ 2 \times 10^{-6} \\ 4 \times 10^{-6} \end{array} $	10 5 18 2 22 2 30 0
9	Antistin HCl ,,,,,, Quinidine HCl ,,,,	10 ⁻⁶ 2 × 10 ⁻⁶ 4 × 10 ⁻⁶ 2 × 10 ⁻⁶ 4 × 10 ⁻⁶ 8 × 10 ⁻⁶	6 9 13 6 29 6 12 5 17 0 27 0
10	Antistin HCl	10 ⁻⁶ 2 × 10 ⁻⁶ 4 × 10 ⁻⁶ 2 × 10 ⁻⁶ 4 × 10 ⁻⁶ 8 × 10 ⁻⁶	3.2 16 0 21 5 10 9 12 5 15 3
11	Antistin HCl ,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	10 ⁻⁶ 2 × 10 ⁻⁶ 2 × 10 ⁻⁶ 2 × 10 ⁻⁶ 4 × 10 ⁻⁶ 8 × 10 ⁻⁶	3 6 6 4 12 8 3 9 7 0 12 5
12	Antistin HCl "" Quinidine HCl "" ""	2 × 10 ⁻⁶ 4 × 10 ⁻⁶ 8 × 10 ⁻⁶ 2 × 10 ⁻⁶ 4 × 10 ⁻⁶ 8 × 10 ⁻⁴	9 5 13 7 16 4 9 0 12 5 17 4

Antagonism to acetylcholine on skeletal muscle

These observations were first made on the skeletal muscle of the frog A strip from the frog's (Rana temporaria) rectus abdominis was suspended in the oxygenated frog-Ringer solution in a 5 ml bath at room temperature. The solution was replaced every three minutes by the frog-Ringer solution containing a known concentration of acetylcholine which was allowed to act for 90 sec. Benadryl (Fig. 4) reduced the stimulant action of acetylcholine on the muscle. Similar results were obtained with antistin. The response to 10-5 acetylcholine was considerably diminished after exposure of the muscle to 10-4 antistin for 3 min.

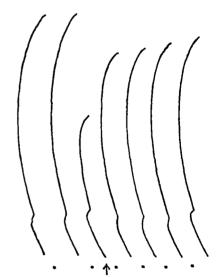


FIG 4—Frog's rectus abdominis in frog-Ringer solution at room temperature. The contractions are due to 2×10^{-6} acetylcholine for 90 sec every 3 min. The contraction at the arrow was preceded by 30 sec exposure to 2×10^{-5} benadryl

The effects of these substances were also studied on the isolated phrenic-diaphragm preparation of the rat (Bülbring, 1946) The technique followed was the same as described in the previous paper (Dutta, 1949) The action of antistin is shown in Fig. 5. In a concentration 4×10^{-5} , antistin increased the height of contractions elicited by single, maximal nerve stimulation by about 20 per cent (Fig. 5a). After the preparation had been washed out, antistin produced the same effect when direct stimulation was applied (Fig. 5b). After the preparation had been washed out again, the addition of 100 μ g of d-tubocurarine chloride to the bath

abolished the response of the muscle to nerve stimulation (Fig. 5c) but not to direct stimulation (Fig 5d), the addition of the same amount of antistin to the bath was followed by increased muscle twitches as before Before the wash-out (W), the stimulation of the nerve had no effect contractions began to return some time after the Tyrode solution had been changed again (W) This is shown on the right-hand side of the record (Fig. 5d) higher concentrations antistin caused a curare-like depression of the rat diaphragm The minimum concentration necessary to demonstrate this varied with different preparations In the experiments illustrated in Fig 6B, the effect of antistin in a concentration

 16×10^{-4} was most pronounced Depression of the muscle was observed with only one quarter of this concentration in other experiments. When the concentration of antistin was high enough to cause marked diminution of the twitch tension of the rat diaphragm, the initial stimulatory stage was seldom missed. However, this was barely evident in the experiment reproduced in Fig. 6B

The action of benadryl was very similar to antistin. This substance in a concentration 4×10^{-5} augmented the contractions of the rat diaphragm by 78 per cent on indirect stimulation and by 50 per

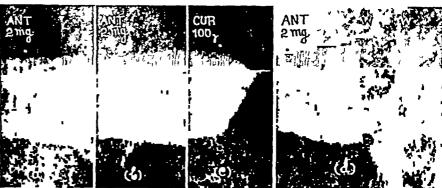


Fig 5—Rat diaphragm phrenic nerve preparation in Tyrode solution, 37° C, 50 ml bath Single, maximal shock, 7 per min, 0.7 millisecond Tyrode solution changed between (a) and (b) and between (b) and (c) (a) Indirect stimulation 2 mg antistin increased the muscle contractions (b) Direct stimulation 2 mg antistin had the same effect as in (a) (c) Indirect stimulation progressive paralysis of the muscle to nerve stimulation after 100 μg d-tubocurarine chloride (d) Direct stimulation 2 mg antistin still enhanced the contractions Before being washed out (W) the muscle gave no response to indirect stimulation, but the contractions began to return after the second washing out, as shown in the right-hand side of the record

cent when the muscle was stimulated directly After the diaphragm had been made insensitive to nerve stimulation by the addition of 100 μg of d-tubocurarine chloride to the bath, the same concentration of benadryl still increased the muscle twitches in response to direct stimulation by 23 per cent. In these experiments the rate of stimulation was 7 per min, the duration of each stimulus being 0.7 millisec Benadryl in a concentration 4×10^{-5} also caused depression of the contractions in response to nerve stimulation. This decline in the height of muscle twitches was usually preceded by a period of augmentation (Fig. 6D)

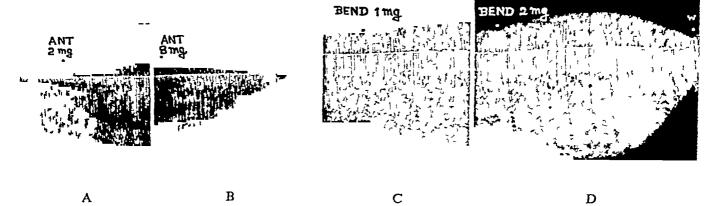


Fig 6—Rat diaphragm phrenic nerve preparation in Tyrode solution, 37° C, 50 ml bath A and B single, maximal nerve stimulation, 7 per min, 0.5 millisecond. The solution was changed between A and B, 2 mg antistin increased the contractions (A) while 8 mg produced depression of the muscle tension (B) preceded by a very short stage of stimulation. C and D single, maximal nerve stimulation, 6 per min, 1 millisecond. The solution was changed between C and D, 1 mg benadryl produced an increase in the amplitude whereas 2 mg caused an initial increase followed by progressive depression till the bath was washed out (W)

Action on the perfused superior cervical ganglion

From the results of the previous experiments a suggestion arose that the curare-like action of these substances on the skeletal muscle might be exerted by blocking the neuromuscular transmission led to an examination of the effect of benadryl and antistin on the perfused superior cervical ganglion of the cat The procedure was the same as described in the earlier paper (Dutta, 1949) The ganglion was perfused with Locke's solution at 35° C Fig 7 (upper record) the depressant action of benadryl is illustrated The record shows the contractions of the nictitating membrane in response to preganglionic stimulation (16 per sec, every 3 The third stimulation was preceded by a dose of 250 µg benadryl and the response to the next stimulation was abolished The ganglion recovered gradually The effect of antistin is shown in Fig 7 (lower record) and the results of all the experiments are summarized in Table IV

TABLE IV

ACTION ON SUPERIOR CERVICAL GANGLION OF THE CAT

Exp No	Drug	Amount injected in µg	Height traction nictitatin brane i	Percen- tage inhibi-	
		m 25	Before addition of drug	After addition of drug	tion
2 3 4 5 6 7 7 8	Benadryl	100 200 400 400 300 400 600 250	18 65 51 36 30 42 40 47	3 0 37 21 25 34 0	83 100 27 42 17 19 100 100
4 5 5 6 8	Antistin	500 200 400 600 200	40 52 52 28 53	29 48 26 18 39	28 8 50 36 26

Effect on histamine-induced gastric secretion

There has been some difference of opinion about the effect of benadryl on the gastric secretion evoked by histamine Experiments were therefore carried out on cats by the method described by Howat and Schofield (1948) A cat, which had been kept without food overnight, was anaesthetized with chloralose and a rubber tube was inserted in the pylonic end of the stomach for withdrawal of the gastric juice The stomach was washed out with

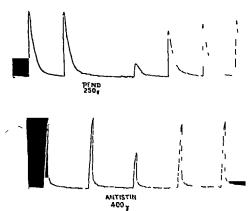


FIG 7—Superior cervical ganglion of the cat, perfused with Ringer-Locke's solution 'Contractions of the nictitating membrane in response to preganglionic stimuli, 16 per sec for 10 sec every 3 min Upper record response to stimulation was abolished after 250 µg benadryl The ganglion recovered shortly Lower record response to stimulation was diminished after 400 µg antistin

warm water through an opening made in the oesophagus which was reached by an incision in the neck of the animal This was later closed cat was warmed so as to maintain a constant rectal temperature Atropine sulphate (1 mg/kg) was injected intravenously to reduce the effect of vagal impulses After an hour the stomach was washed with N/200 HCl and 25 ml left in Every 15 min the stomach was emptied and refilled with 25 ml of fresh dilute solution of the acid The amounts of free and total acid present in each sample were estimated by titration with N/20 NaOH, thymol blue being used as indicator The samples drawn out immediately before each histamine infusion were taken as the initial level for each secretory curve Histamine acid phosphate (0 0045 mg/kg/min) in normal saline was infused intravenously for 45 min by a slow infusion pump The total amount of acid secreted during this period constituted the first After the secretion had returned to the basal level a second infusion of histamine was given and the total amount of acid secreted formed the Benadryl was injected intravensecond response ously before the beginning of the second histamine administration

The secretion of free hydrochloric acid rose steadily during the infusion of histamine and declined progressively as soon as the infusion ended. It returned to the basal level within an hour. The figures for total and free acid were very close to one another. The amount of acid secreted during each response is expressed throughout as millilitres of

TABLE V

DOUBLE HISTAMINE TEST ON GASTRIC SECRETION IN CATS (AFTER ATROPINE)

1st and 2nd responses = total acid secreted during 1st and 2nd infusions of histamine respectively

	Controls			Benadryl 3 mg /kg between 1st and 2nd responses			
Exp	Wgt kg	1st response N/20 HCl ml	2nd response N/20 HCl ml	Exp	Wgt kg	1st response N/20 HCl ml	2nd response N/20 HCl ml
1 2 3 4 5 6 7 8 9	3 2 3 2 3 3 3 3 2 5 2 5 4 9 4 5 3 4 3 0	10 2 3 3 17 7 10 4 6 5 9 6 21 6 10 3 20 4 12 2	21 1 9 3 11 7 11 4 9 3 22 8 14 1 9 8 18 9 10 0	1 2 3 4 5 6 7 8 9	3 2 5 5 5 3 3 2 8 3 0 3 2 2 8 3 0 2 8 3 0	8 2 18 2 12 0 7 5 7 9 4 8 6 8 8 1 6 1 17 9	13 9 18 1 13 8 31 8 16 0 11 9 18 4 14 1 12 0 10 3
Mean		12 22	13 84	Mean		9 75	16 03
Mean difference $p = 0.5$, n	Mean difference 1.6 ± 2.2 , $t = 0.73$, $\pm \text{Std}$ error $p = 0.5$, not significant		Mean differ	rence 6 28 : than 0 05, s	± 266 , $t = 236$, ± Std error	

N/20 HCl When these figures are compared (Table V) the variation between the responses of individual cats becomes obvious. In four of the ten control experiments (Table V), in which no drug was administered between the two injections of histamine, the output of acid during the second response was greater than the first, in another four cats the first response was greater than the second, and in the remaining two no appreciable difference between the two responses was noticed. The mean total amount of acid secreted during the first response (Table V) was 12.2 ml and during the second res-

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FIG 8—Gastric acidity in atropinized cats, double histamine test. Mean of ten control experiments in which no drug was administered between the two histamine responses. Histamine acid phosphate (0 0045 mg /kg /min) was perfused intravenously for 45 min each time. The mean second response showed an increase by 13 0 per cent over the first. This was not statistically significant.

ponse it was 13 8 ml, the mean difference, which was 16 ml, was not statistically significant. Fig 8 shows graphically the mean results of ten control experiments. In the experiments with benadryl, the mean titratable acidity of the second response was greater than the first by 63 3 per cent (Table V). Since t=236, the value of p was less than 005, i.e., the second response differed from the first significantly. Thus after benadryl the gastric cells secreted a greater amount of acid in response to histamine than before the administration of the antihistamine substance.

DISCUSSION

Benadryl is reputed to possess an atropine-like action, for it reduces the depressor response of acetylcholine in the dog (Loew, MacMillan, and Kaiser, 1946), antagonizes the stimulant effect of acetylcholine on the gut (Loew, MacMillan, and Kaiser, 1946), causes mydriasis, and alters accommodation (Harris, McGavack, and Elias, 1946) Antistin also prevents the contractions of the isolated intestine induced by acetylcholine (Meier and Bucher, 1946) The effect of benadryl and antistin in inhibiting the action of acetylcholine on the isolated rabbit auricles is additional evidence in this direction

Dawes (1946) reported that the substances which prolong the refractory period of cardiac muscle are antagonists of acetylcholine. This is also true for benadryl and antistin, for both of them reduced the

maximal rate of the electrically stimulated rabbit auricles. This effect is not due to the generalized depression of the auricular tissue. If doses of benadryl and antistin which are sufficient to reduce the maximal rate of contractions of electrically driven auricles are added to a bath in which isolated rabbit auricles are beating freely, the amplitude of contractions is increased. It is necessary to raise the concentration several times before any reduction of the height of contractions or slowing of the organ is noticeable.

Acetylcholine increases the maximal rate of contraction of the driven auricles (Dawes, 1946, Elfo, 1947), this effect is increased by eserine or physostigmine and abolished by atropine (Elío, 1947) Elio has further suggested that "the transmission of the impulse in cardiac muscle may be effected by a mechanism in which acetylcholine is a key substance" Recently, Bülbring and Burn (1949) showed that the isolated rabbit's auricle is canable of synthesizing acetylcholine, and that the contractions of an auricle, which has been allowed to beat continuously until it has stopped, can be started again by the addition of acetylcholine to the bath If, therefore, one assumes that acetylcholine takes part in transmission of cardiac impulses, it is not difficult to explain the action of benadryl and antistin in reducing the maximal rate of the rabbit's auricle, for both of them share the property of opposing the action of acetylcholine at various sites

On the skeletal muscle of the rat diaphragm these substances produced a dual effect. In small concentrations they increased the response to a single maximal electrical shock, but in higher doses the opposite effect is produced. The evidence is strong that both benadryl and antistin act on the muscle directly, since the augmentation of the muscular twitches caused by indirect stimulation was also seen in response to direct stimulation and was not affected by d-tubocurarine chloride Both benadryl and antistin prevented the contractions of the nictitating membrane in response to preganglionic sympathetic stimulation in the perfused ganglion It may be that the diminution of the twitch tension of the rat diaphragm in response to nerve stimulation when under the influence of these substances is at least in part due to some blocking of the neuromuscular transmission

In three of their four dogs with vagal denervated gastric pouches, Loew, MacMillan, and Kaiser (1946) noticed a significant decrease of the acid secretion in response to histamine after benadryl Using about three times the dose employed by the previous workers, Friesen, Baronofsky, and Wangensteen (1946) failed to observe any such effect.

Sangster, Grossman, and Ivy (1947) also did not notice any evidence which justified the conclusion that benadryl diminished the response of the gastric glands to histamine in dogs Studies on man (Moersch, Rivers, and Morlock, 1946, McElin and Horton, 1946) did not provide any definite evidence that benadryl antagonized the action of histamine on the gastric secretory response. On the contrary. in some subjects the output of acid was actually stimulated (Gilg, 1948, Ashford, Heller, and Smart, The results of the present investigation 1949) clearly show that after benadryl the secretion of titratable acid from the stomachs of atropinized cats is usually increased in response to histamine Recently Bain, Broadbent, and Warin (1949) have shown that the early effect of the antihistamine substance phenergan is to potentiate the reaction to an intradermal injection of histamine

SUMMARY

- Both antistin and benadryl =
- (1) when tested by the intracutaneous weal method in the guinea-pig, show a linear relationship between the logarithmic concentration of the substances and their local anaesthetic effect. Both are more potent than procaine,
- (11) reduce the depressant action of acetylcholine on the isolated rabbit's auricle,
- (ni) decrease the maximal rate at which the isolated rabbit's auricle will respond to electrical stimulation. The relation between the percentage decrease in the maximal rate and the logarithm of the concentration is linear for both these substances. Both of them are more active than quinidine.
- (1v) reduce the contractions produced by acetylcholine on the isolated frog's rectus,
- (v) in small concentrations increase the twitch tension of the rat's diaphragm elicited by single, maximal nerve volleys. These enhanced muscular contractions are also seen in response to direct stimulation in the curarized preparation. When concentrations of these substances are raised, a decline in the muscle's response to indirect stimulation follows,
- (vi) depress the contraction of the nictitating membrane in response to preganglionic stimulation when injected into the fluid perfusing the superior cervical ganglion
- 2 In cats after atropine, benadryl significantly increased the rate of hydrochloric acid secretion from the stomach during intravenous perfusion of histamine

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THE PRESENCE OF ACETYLCHOLINE IN TRYPANOSOMA RHODESIENSE AND ITS ABSENCE FROM PLASMODIUM GALLINACEUM

Β'n

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The finding in this laboratory (Burn and Vane, 1948) that acetylcholine is capable of restoring the spontaneous contractions of isolated rabbit auricles after they have been stopped by the antimalarial substance proguanil raised the question of the possible importance of acetylcholine for pathogenic protozoa in general. The further demonstration of a close relationship between the motor activity of auricular muscle and its ability to synthesize acetylcholine (Bülbring and Burn, 1949) suggested the possibility that this substance might be of particular importance to highly motile organisms such as trypanosomes

I ACETYLCHOLINE IN Trypanosoma Rhodesiense

(a) Identification of acetylcholine

The trypanosome used was an old strain of *T rhodesiense* which has been maintained at the Liverpool School of Tropical Medicine for the past 26 years. It causes a fulminating septicaemic type of infection in mice and rats, ending fatally three or four days after a light inoculation.

Preliminary experiments confirmed that washed rat blood cells neither contain, nor synthesize under the most favourable conditions, any measurable amount of acetylcholine

For these experiments rats were bled into 1 per cent (w/v) sodium citrate in normal saline solution containing 1 10,000 eserine, the blood cells then being centrifuged, resuspended in eserine-saline, and again centrifuged. The cells thus packed were present in concentrations ranging, in individual experiments, up to 17,800,000 per cu mm A mixture was made of equal parts of this packed blood cell material and a substance described by Feldberg and Mann (1946) as "activator," prepared by extracting an acetone-dried powder of rabbit brain with hot saline, and found by these workers to enhance the synthesis of acetylcholine by brain tissue. The mixture was then divided into three portions of 2 c c, each One of these was acidified with 1 c c N/3 HCl and boiled in order to destroy any possible choline acetylase present, the second portion received no preliminary treatment, while

the third was converted into an acetone-dried powder by washing several times in acetone and drying in a vacuum desiccator, the powder being then suspended in 2 c c of "activator" The three specimens (2 c.c each) were next incubated for 75 min at 37° C with the ingredients (and in the proportions) shown by Feldberg and Mann (1946) to be suitable for the synthesis of acetylcholine by brain tissue and used also by Bulbring and Burn (1949) for acetylcholine synthesis by rabbit auricles specimens, duly neutralized by the addition of N/3 NaOH, were then examined for acetylcholine by their effect on the frog rectus muscle Any acetylcholine which might be shown to be present in the first specimen (acidified and boiled before incubation) must necessarily have been present from the beginning, any difference in content between the first and second specimens must represent synthesis that had taken place during the period of incubation, whilst acetylcholine shown to be present in the third specimen (the acetone-dried powder) must also have been synthesized during incubation acetylcholine could, however, be demonstrated in any of the specimens Since these tests showed that rat blood cells neither contain hor synthesize acetylcholine, it was accordingly held to be unnecessary to separate blood cells from trypanosomes when preparing a suspension of trypanosomes for investigation of their possible acetylcholine content The trypanosome material was therefore prepared in the following way

Six rats were inoculated intraperitoneally (0.3 c.c. per rat) with heart's blood obtained from 2 very heavily infected mice Two days later the blood of these rats, in turn, was swarming with parasites, and 36 cc were obtained by heart puncture and added to 108 c-c of sodium citrate-saline solution (1 g Na₃C₆H₅O , 2H₂O/100 cc, 085 g NaCl/100 cc) Eserine, 288 cc of a solution containing 0 5 g eserine sulphate/100 c c was immediately included, giving a concentration of 1 10,000 eserine in the trypanosome-blood mixture This was then centrifuged at 5,500 rpm for 2 min, the supernatant replaced by saline solution containing 1 10,000 eserine, the mixture again centrifuged at 5,500 rpm for 2 min, and the supernatant then discarded The deposit, consisting of densely packed blood cells and trypanosomes, occupied 14 4 c c To this were added an equal volume of saline and 72 cc N/3 HCl, the mixture then being rapidly boiled and cooled, brought

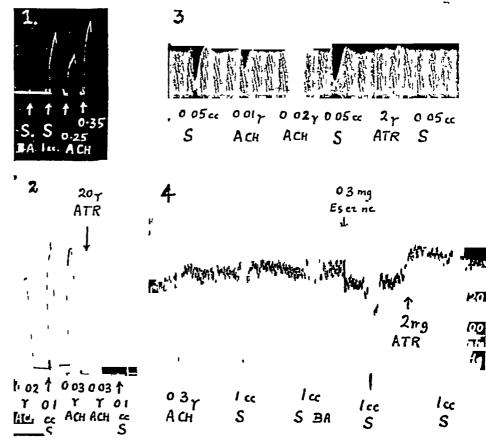
to 72 0 c c in frog-Ringer solution, and stored in the ice-box for determination of acetylcholine the following day

The material was neutralized and then assayed by four separate methods, two depending on motor and two on inhibitor effects characteristic of acetylcholine. The motor tests were on the isolated frog rectus muscle and the isolated guinea-pig ileum, and the inhibitor tests on the perfused isolated frog heart and the blood pressure of the spinal cat

Results were as follows

- (i) Contraction of the isolated frog rectus muscle (see Fig 1) In order to deal with the possible complication of non-specific contractions produced by the trypanosome material, a portion of the 72 c c obtained as above was treated with N/3 NaOH and boiled to destroy any acetylcholine that might be present. After cooling and neutralizing with N/3 HCl, this control specimen (SBA) failed to cause any contraction of the rectus muscle. By adding known amounts of acetylcholine to portions of the control specimen, and matching the contractions which these produced against the contractions produced by untreated portions of the test fluid, 1 c c of this fluid (S) was found to correspond to 0.3 μ g acetylcholine
- Fig 1—Contractions of the frog rectus muscle SBA = specimen of trypanosome material boiled in alkali, cooled, and neutralized S = specimen not so treated 10 c c of S equivalent to 03 µg acetyl-choline (ACH)
- FIG 2—Contractions of guineapig ileum 01 c c of trypanosome material(S) equivalent to 003 μg acetylcholine (ACH) Effect abolished by 20 μg atropine (ATR)
- Fig 3—Perfused isolated frog heart beat 0.05 c c of try-panosome material (S) equivalent to 0.015 μg acetylcholine (ACH) Effect abolished by 2 μg atropine (ATR)
- FIG 4—Blood pressure of spinal cat 10 c c of trypanosome material (S) equivalent to 03 μg acetylcholine (ACH) No effect produced by 10 c c trypanosome material previously boiled in alkali, cooled, and neutralized (SBA) Effect of 1 c c S enhanced by 0 3 mg eserine and abolished by 2 mg atropine (ATR)

- (ii) Contraction of the isolated guinea-fig ileum (see Fig 2) The contractions produced by 0 1 c c of the trypanosome material were found to be equal to those produced by 0 03 μ g acetylcholine, thus agreeing with the result obtained by the frog rectus method above That it was acetylcholine which was responsible for the contractions produced by the trypanosome material is supported by the fact that the contractions could be prevented by atropine
- (iii) Perfused isolated frog heart beat (see Fig 3) The diminution which 0 05 c c of the test specimen produced in the amplitude of the heart beat was the same as that produced by 0 015 μ g acetylcholine, thus agreeing quantitatively with the results obtained by the two motor tests above. Again the effect could be prevented by atropine
- (iv) Blood pressure of spinal cat (see Fig 4) The blood pressure was reduced to the same extent by 1 c c of the test specimen as by 0 3 μ g acetylcholine, thus again agreeing quantitatively with the results obtained by the other methods. No fall in blood pressure was produced by the specimen after it had been boiled with alkali (SBA). The fall was increased by prior injection of eserine and abolished by atropine



The fact that the trypanosome material produced effects characteristic of acetylcholine in each of these four tests, and that, moreover, its acetylcholine content was in perfect agreement (0 3 µg per c c.) for each assay method, leaves no room for doubt that it was indeed acetylcholine and not some unidentified substance which was responsible for the reactions observed

(b) Synthesis of acetylcholine

The possibility seems remote that the trypanosomes absorb preformed acetylcholine from some organ or body fluid of the host. It would be much more likely that they acquire the substance by their own powers of synthesis, and this we have been able to demonstrate. After a few orientating experiments, the following routine procedure was adopted in the preparation of trypanosome material for this further work.

Four rats were inoculated intraperitoneally with heavily infected mouse blood on which a trypanosome count had previously been carried out, so that the amount injected per rat could be adjusted to contain about 2 × 10^s trypanosomes. Two days later the rats were sufficiently heavily infected for the required purpose. It was found advisable to use rats at levels of infection thus obtained rather than rats that are even more densely infected, since trypanosomes obtained from the latter tended to disintegrate soon after bleeding, resulting in an underestimate of the amount of trypanosome material present if there should be any undue delay in counting the trypanosomes. The rats were bled by heart-puncture and 30 c c of blood added to 90 c c of 1 per cent sodium citrate-saline solution.

The trypanosomes were counted at this stage, and the total of 120 c.c then divided into two equal portions, to one of which was added 1 2 c c 1/200 eserine (final eserine concentration therefore 0.01 per cent) The reason for treating one portion with eserine was to preserve any acetylcholine that might otherwise be destroyed in the course of the subsequent in vitro operations Each of the two 60 cc portions, non-esermized and esermized respectively (labelled N and E for convenience), was then divided into 3 equal lots of 20 c.c each These were then centrifuged at 5,500 r p m for 2 min, the supernatant discarded and replaced by saline for N and by eserine saline for E, centrifuged once more at 5,500 r p m for 2 min, and the supernatant fluid again discarded, thus leaving 3 specimens of N and 3 of E, each consisting of about 2 c c packed blood cells and trypanosomes The number of trypanosomes in each of the six specimens thus obtained varied in different experiments between 3 04° and 6 37°

The 3 specimens of N and E respectively were then treated in essentially the same way as the 3 specimens obtained from uninfected rat blood in the experiment described on p 250 designed to confirm that the blood cells neither contain nor synthesize acetylcholine, that

is, one specimen was acidified with 1 c.c. N/3 HCl and boiled, one received no such preliminary treatment, and one was converted into an acetone-dried powder

The specimens were then incubated at 37° C with the ingredients and in the proportions described by Feldberg and Mann (1946) and assays again carried out on the frog rectus preparation Standard acetylcholine solutions for matching against the samples of unknown content were made up in the same ingredients as those of the latter samples, except that rat blood cells were not always Tests showed that the omission of this partiıncluded cular component did not affect the result Errors due to contraction of the frog rectus by factors other than acetylcholine were eliminated also by boiling a portion of the unknown solution in the presence of N/3 NaOH, neutralizing, and making up with known amounts of acetylcholine for matching against the unknown Since the blood cells neither contain nor synthesize acetylcholine, any of this substance discovered must have been derived from the trypanosomes, and, again, the difference in acetylcholine content between the specimen acidified and boiled before incubation and the specimen not treated in this way must represent the amount synthesized during the course of incubation Any acetylcholine present in the specimen prepared from the acetone-dried powder must also represent synthesis

The accompanying Table shows the results of a number of consecutive experiments of this type The amounts of acetylcholine have been calculated per 1010 trypanosomes because that number of packed trypanosomes weighs approximately 1 g, the average of three determinations (in good agreement) of the wet weight of trypanosomes separated from blood cells and packed by-centrifugation at 5,500 rpm for 3 min was, in fact, found to be The variations of the results shown in the 0957 g Table are due not merely to experimental error but to the fact that findings are included of the initial orientating experiments of the series before the procedure had been finally standardized as described Thus, the absence of acetylcholine in the specimen without eserine (N) of exp 5 may have been due to the fact that there was an excessive concentration of trypanosomes in the specimen, resulting in their disintegration and the destruction of any acetylcholine that may have been present. Moreover, in these earlier experiments, considerable time was taken up by alternately freezing and thawing the specimens, a procedure which was discarded as unnecessary in the later experiments of the series In the last three experiments, the acetylcholine contents of the preparations without eserine (N)were no longer significantly lower than those of the preparations with eserine (E) The fact that the use of eserine in preparing the specimens for incubation increased the apparent yield of acetylcholine in the earlier experiments affords additional support for

Experiment	Trypanosomes not exposed to eserine before incubation (N)			Trypanosomes exposed to eserme before incubation (E)			
	Cantont	Synthesized during incubation		Content	Synthesized during incubation		
	Content before incubation	Trypanosomes not converted to acetone- dried powder	Trypanosomes converted to acetone- dried powder	Content before incubation	Trypanosomes not converted to acetone- dried powder	Trypanosomes converted to acetone- dried powder	
1* 2a 2b* 3 4 5 6 7 8	3 58 1 86 0 10 3 2 71 5 72	1 80 0 94 0 71 5 58 2 41 2 18	0 0 28 0 1 24	2 67 2 41 2 28 4 54 5 08 8 08 8 23 3 08 6 80	0 0 21 0 0 1 58 1 60 4 23 2 56 1 36	0 60 0 90 0 28 0 0 1 15 0 74	

- TABLE

ACETYLCHOLINE IN Trypanosoma rhodesiense, IN μG PER 1010 TRYPANOSOMES (0 96 G WET WEIGHT)

believing that it is indeed acetylcholine and not some unknown substance with which we are dealing

In two of the earlier experiments (Nos 1 and 2b) the red cells had been carefully separated from the trypanosomes by differential centrifugation before incubation and assay of the latter, but the results as a whole appear to show that there is no advantage in this precaution

Confining our attention now to the eserinized series alone, the mean acetylcholine content before incubation was found to be 4.79 µg (range 2.28 to 8 23 μ g) per 10¹⁰ trypanosomes, or 5 00 μ g. (range 238 to 860 µg) per g wet weight ranged between 0 and 4 23 µg per 1010 trypanosomes in 75 min. In order to compare these figures for synthesis by trypanosomes with those found by Bülbring and Burn (1949) for rabbit auricles we have determined that an acetone-dried powder of 1010 trypanosomes weighs about 0 078 g The amounts synthesized by trypanosomes were therefore 0 to 54 2 μg per g powder (71 5 μg in one result of series N) as compared with 40 2 \pm 12 7 μ g quoted by Bülbring and Burn for rabbit auricles interesting that the figures for trypanosomes and for rabbit auricles should be in such close agreement It might be emphasized, though, that we have not explored the optimum conditions for acetylcholine formation by trypanosomes We have merely used those which Feldberg and Mann (1946) found to be optimal for brain tissue, under other circumstances, as yet unknown, it may well be that much higher figures could be obtained for the activity of trypanosome material in this respect. The Table shows that no particular advantage was gained by preparing an acetone-dried powder of the trypanosome material, and this procedure was therefore omitted in the last two experiments of the series

II LACK OF ACETYLCHOLINE IN Plasmodium Gallinaceum

In order to discover whether the blood stages of *Plasmodium gallinaceum* contain or are capable of synthesizing acetylcholine, the procedure adopted was the same as for *Trypanosoma rhodesiense*, except for minor differences necessitated by differences in the two types of infection. For example, in order to arrive at an estimate of the number of parasites in each specimen, trypanosomes were counted directly in a haemacytometer chamber (after suitable dilution of the specimen), whereas for malaria parasites the total number of red cells was first estimated in the usual way in a counting chamber, the number of parasites then being derived from the ratio of parasites to red cells as determined in stained blood films.

Several experiments were carried out with *P* gallinaceum, the following being a typical example A massive infection was produced in an eight-week-old chicken by inoculating it intravenously with 10 c c blood from another heavily infected chicken Two days after inoculation 22 c c blood was obtained by heart puncture and immediately added to 66 c c sodium citrate-saline solution containing 1 10,000 eserine. The mixture was then divided

^{*} Trypanosomes separated from blood cells before incubation and assay

into 3 equal portions, centrifuged, the supernatant replaced by eserine-saline, and the mixture again centrifuged. The three deposits (containing 9.72° parasites each) were then treated exactly as described above for the investigation of rat blood and try-panosomes, that is, one specimen was acidified with 1 cc N/3 HCl and boiled, one received no such preliminary treatment, and one was converted to an acetone-dried powder. The specimens were then incubated in the manner described above, and tested on the frog rectus preparation. No evidence of acetylcholine could be detected in any of the specimens tested.

DISCUSSION

Since the isolation by Ewins (1914) of acetylcholine from a liquid extract of ergot, implying its production by *Claviceps purpurea*, very little attention has been directed toward the possibilities of acetylcholine formation by other micro-organisms. A recent and convincing contribution in this field has been the demonstration by Stephenson and Rowatt (1947) of acetylcholine production by a strain of *Lactobacillus plantarum*, from sauerkraut Bayer and Wense (1936) showed acetylcholine to be present in a species of the free-living protozoon Paramoecium, but there seems to have been no investigation till now of the production of acetylcholine in pathogenic protozoa

It is not possible at the present stage to do more than speculate, in seeking for an interpretation of our finding that trypanosomes synthesize and presumably utilize acetylcholine while malaria parasites do not It may, however, be relevant that acetylcholine is well known to be intimately concerned in a variety of motor mechanisms among higher organisms ranging from some of the invertebrates (e.g., the leech) up to the highest forms of vertebrate life Its importance for motor processes has recently been suggested even in a location where it was formerly regarded as essentially an inhibitor substance, namely, in heart muscle, for the work of Bülbring and Burn (1949) provided evidence for believing that the activity of auricular muscle is inseparably linked with its synthesis of acetylcholine One is therefore tempted to correlate the presence of acetylcholine in trypanosomes and its absence from malaria parasites with the fact that the former are highly active motile organisms while the latter are relatively immobile. This reference to the lack of motility on the part of malaria parasites should, of course, be qualified by recognizing, firstly, that it is only the blood stages and not the mosquito phases

with which we are here concerned, and, secondly, that slight motility, varying in degree among different species of malaria parasite, is in fact exercised during the trophozoite (or amoeboid) stage of development Indeed, the specific name Plasmodium vivax testifies to the prominence of this particular feature in the parasite of benign tertian malaria. in comparison with other species of malaria parasite The motility is, however, relatively sluggish, being essentially amoeboid in type and quite unlike that of trypanosomes with their highly specialized organs of locomotion The demonstration by Bayer and Wense (1936) of acetylcholine in Paramoecrum may be significant in this connexion since Paramoecium 15. of course, also characterized by morphological differentiation providing for a high degree of motility

It need hardly be added that we do not attempt to assign to acetylcholine some role in connexion with high motility as its sole possible function among micro-organisms. Its presence in *Claviceps purpurea* and *Lactobacillus plantarum* is alone sufficient to indicate that this cannot be the case. Likewise, we do not exclude the possibility that a high degree of motility may occur in some species of protozoa or related organisms without acetylcholine being involved. Among the lower orders of metazoa Bacq (1947) points out that coelenterates and tunicates, which are far from devoid of motor properties, contain either no acetylcholine or only inappreciable amounts of that substance.

SUMMARY

- 1 Trypanosoma rhodesiense has been found to contain acetylcholine to the extent of 2 28 to 8 23 μ g per 10¹⁰ trypanosomes, or 2 38 to 8 60 μ g per g wet weight. The formation of acetylcholine has been demonstrated in vitro in amounts up to 5 50 μ g per 10¹⁰ trypanosomes, or 71 5 μ g per g acetone-dried powder, in 75 min at 37° C
- 2 Plasmodium gallinaceum has been found neither to contain nor to synthesize acetylcholine

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THE FATE OF TUBOCURARINE IN THE BODY

BY

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The need for isolating a purified active principle from crude curare started at an early date in South America, when Boussingault and Roulin (1828) succeeded in obtaining a bitter principle which they differentiated from strychnine, isolated eight years Although the problem was somewhat previously clarified by the work of Preyer (1865) and Boehm (1886, 1897), it was not until 1935 that the active alkaloidal salt, dextro-tubocurarine chloride, was isolated in a pure crystalline state by King from a sample of native tube-curare The same alkaloid was obtained in a good yield by Wintersteiner and Dutcher (1943) from a single plant species, chondrodendron tomentosum, which is probably its chief botanical source

The fate of this alkaloid in the body has received little attention, the present work is an attempt towards providing some information on this point

CHOICE OF A METHOD

The methods of assaying curare-preparations in general are either chemical or biological

Chemical methods—Barbosa (1903) published elaborate charts depicting the colours obtained with various curare compounds. Qualitative, though non-specific, reactions for the curare alkaloids with potassium ferrocyanide were described by Cole (1923) and with trichloracetic acid by Schoofs (1927). Recently, Foster and Turner (1947) have developed a tentative polarimetric method for the assay of d-tubocurarine chloride. They also described a colorimetric method for the assay of the alkaloid depending on the use of Folin-Ciocalteu phenol reagent.

All these methods, however, must be of limited application on account of the many materials which yield colours with the reagents, and the high concentration of the alkaloid required for its detection

Biological methods —One of the early methods was described by Gaddum (1937) and consisted in determining the paralysing dose for the frog Holaday (1941) developed the head-drop method,

which employs muscular relaxation in an intact mammal (rabbit) as the criterion of curare activity. The intravenous injection of curare in mice likewise produces a head-drop. This method, described by Kimura and Unna (1948), is claimed to be more economical and allows the statistically valid determination of the head-drop dose on a uniform population. In this connexion it may be noted that the difference between the average head-drop dose and the average lethal dose represents the margin of safety of the drug, and that this margin is so narrow that the determination of the average head-drop dose often results in loss of the animal. Moreover in a head-drop method the results are not recorded objectively

Skinner and Young (1947) described a "mousemethod" of assay of curare activity as being simple and objective Marsh and Pelletier (1948) used a method depending on comparing the paralytic doses for rats

The main use of any of these methods at present is mostly confined to the evaluation of compounds with curare-like activity, and even there they suffer from the disadvantage that they do not establish the site of action of the drug, which is one of its most characteristic features

The isolated frog's nerve-muscle preparations (gastrocnemius-sciatic or nerve-sartorius) have also been used, most authors (for references, see Ing, 1936) have estimated either concentrations which paralysed the muscle completely or which just failed to cause complete paralysis. After such severe poisoning, recovery is usually slow

Chou (1947) used the rat's phrenic nervediaphragm preparation (Bülbring, 1946) for the estimation of curare-like activity. The use of this method for assay requires the presence of the test substance in relatively large concentrations

The frog's rectus abdominus muscle method

This method depends on a measurement of the antagonism between acetylcholine and tubocurarine, and although it is probably more sensitive than other

methods it is not specific. It was described by Jalon (1947) and was found suitable for the purpose of the present work. The bath used here for this preparation contains 2 ml Ringer's solution at room temperature, aerated by a continuous stream of oxygen bubbles, and the contractions are recorded on a slowly moving smoked drum. The magnification is about 10 and the tension about 3 g weight A suitable fixed dose of acetylcholine is added to the bath every 6 min and allowed to act for exactly 2 min before it is changed and the preparation allowed to relax When the responses of the rectus muscle to acetylcholine have become quite regular. a suitable volume of the solution to be tested is added 90 sec before the addition of acetylcholine. and its effect on subsequent responses to the latter observed When doses are thus added at a constant time interval, the effect produced in the given constant time is regularly related to the dose and can be taken as an index of the potency of the solution Thus a quantitative estimate of the amount of tubocurarine present in an extract is obtained by comparing it with a standard solution of tubocurarine. given in alternate doses By this procedure it is possible to detect 0.1 µg tubocurarine. As it made no difference to the assay whether or not the rectus had been sensitized beforehand by eserine, the unesermized preparation was preferred

THE PREPARATION OF EXTRACTS

In extracting added or injected tubocurarine from the various tissues and biological fluids, the following methods were tried

Blood -Using whole blood in vitro, most of the drug added was recovered from the plasma by the use of acid alcohol Extracts of blood cells gave no evidence that tubocurarine had passed inside them After an injection, extracts were made in the following way The blood was mixed with heparin and cooled with ice, and the plasma immediately separated The acid alcohol was prepared by acidifying absolute ethyl alcohol with a crystal of tartaric acid or with 0.1 ml NHCl, and 10-15 ml were used for each 1 ml plasma The plasma was added dropwise to the acidified alcohol, which was shaken thoroughly after each addition The precipitate was separated and washed with acid alcohol, the alcoholic solution taken down to dryness on a water bath, and the residue dissolved in Ringer's solution and filtered

Urine —A certain volume of the urine was evaporated to dryness on the water bath. The solid residue was then thoroughly mixed with absolute ethyl alcohol (5 ml for each 1 ml urine) and the precipitate separated by centrifuging and washed with absolute alcohol. The alcoholic solution was then evaporated to dryness, and the final residue taken up in Ringer's solution (1–10 ml for each 10 ml urine), thus the tubocurarine in the urine could be concentrated 1–10 times

Tissue—A convenient method was found to depend on extraction with acid alcohol, and here the use of sulphuric acid as an acidifying agent was usually found to be superior to hydrochloric acid in providing a final clear extract, this was observed by Chang and Gaddum (1933) when they were estimating the acetylcholine equivalent of tissue extracts. Since the acetylcholine in these extracts would interfere with the test for tubocurarine it was inactivated by hydrolysis. Extracts prepared in this way may contain other pharmacologically active substances, and are therefore only suitable for use in a biological test, which is relatively little affected by these substances, this is another advantage of the frog's rectus muscle which is insensitive to most substances in extracts except acetylcholine.

The tissue was weighed, cut up with scissors, and mixed with acidified alcohol (15–20 ml per g tissue), where its cutting up and mixing were completed. This acidified alcohol was prepared by adding 1 2 ml $2N H_2SO_4$ to each 100 ml of absolute ethyl alcohol. The deposit was then separated by centrifuging and washed with acid alcohol, the alcoholic solutions were evaporated to dryness and the residue taken up in Ringer's solution and filtered. The extract was then made slightly alkaline and boiled for 1–2 min to destroy acetylcholine but not tubocurarine, finally it was neutralized and concentrated until 1 ml corresponded to 1–5 g tissue

Faeces—In some animal experiments it was desired to look for the presence of the drug in the faeces. Here, the masses were powdered and a weighed quantity transferred to a dry clean mortar and thoroughly mixed with absolute ethyl alcohol (10 ml per g), the deposit was separated and washed with alcohol, and the alcoholic solutions finally evaporated to dryness and the residue dissolved in Ringer's solution and filtered

Gastric juice —In the conscious human subject, excretion of the drug was sought for in the saliva and gastric juice. The fasting gastric juice, aspirated through a Ryle's tube, was well shaken and filtered. The filtrate was heated on the flame and the coagulum separated, the clear fluid was neutralized and used for the test

Saliva —To each 9 ml absolute alcohol, acidified by few drops of dilute HCl, 1 ml saliva was added drop-by-drop, the alcoholic solution being shaken thoroughly after each addition and for sufficient time at the end The thin precipitate was separated and washed with acid alcohol The alcoholic solutions were evaporated to dryness and the residue taken up in Ringer's solution and filtered

It may also be mentioned that in extracting biological fluids a control sample of the fluid was always obtained before the injection of the drug was made, and was extracted and examined in the same way as the later samples. When the samples obtained after the injection showed measurable curariform activities, this usually decreased as the time interval after which the sample had been obtained became longer, until it faded away approaching the blank control

Such an activity was assumed to be due to the presence of the drug in the corresponding fluid. As for the tissues, a control extract was prepared from the corresponding

tissue of a control animal The control biological fluids and tissue extracts thus obtained were devoid of curariform activity All extracts were, if necessary, made neutral before they were used in the test

RESULTS

In order to test the accuracy of the methods used, the recovery of known amounts of *d*-tubocurarine chloride added to the various tissues and biological fluids was tried. Table I shows that the recoveries of known amounts added to blood, urine, and saliva were satisfactory. In Table II the recoveries of known amounts added to the various tissues are recorded.

TABLE I RECOVERY OF TUBOCURARINE ADDED TO BIOLOGICAL FLUIDS

Biological fluid	Tubocurar tration	me concen- μg /ml	Per cent loss	
nuid	Added Recovered		1055	
Human blood Rabbit's ,,	2 0 1 0 1 0 2 0 1 0 1 0	1 80 0 90 1 0 1 9 0 85 1 12	+10 +10 0 +5 +15 -12 Mean + 46	
Human urine Rabbit's ,, Rat's ,,	2 0 1 0 2 0 1 0 1 0 1 0	1 9 1 0 1 90 1 15 1 0 0 90	+5 0 - +5 -15 0 +10 Mean + 0 83	
Human salıva	2 0 1 0 1 0	2 10 0 85 0 85	-5 +15 +15 Mean + 8 3	

TABLE II
RECOVERY OF TUBOCURARINE ADDED TO TISSUES

Tissue	Tubocura tration	nne concen- μg/g	Per cent	
	Added	Recovered	loss	
Minced mouse	1 0 1 0 0 5	1 10 1 05 0 45	-10 -5 +10	
Rabbit's liver	1 0 1 0	1 07 0 9	-7 +10	
Rabbit's muscle	0 5 0 5	0 45 0 4	+10 -20	
Rabbit's kidney	0 5 0 4	0 45 0.35	+10 +12 5 Mean + 5 6	

The fate of tubocurarine in man

This was studied in five subjects, one conscious man and four patients undergoing surgical operations and kept under cyclopropane-oxygen anaes-The study was made by determining the blood levels of the drug at various intervals after its intravenous administration, by determining the amount excreted in the urine and in the conscious subject, by detecting and estimating the drug in some other biological fluids 1 e, the saliva, gastric juice, and cerebrospinal fluid All control samples were collected before the injection, then the drug. tubarine "BW," was injected intravenously in a dose of 0 2 mg/kg The patients were in the second plane of anaesthesia Blood samples were drawn on the third, fifteenth, and thirtieth minutes after the injection Urine specimens were collected at hourly intervals after the injection. Furthermore, in the conscious subject, a sample of saliva was collected on the twenty-first minute The spinal canal was tapped and a sample of cerebrospinal fluid drawn thirty-three minutes after the injection, and ten minutes later a specimen of the fasting gastric juice was aspirated

In Fig 1 the average levels of the drug in the blood are presented From this curve it may be noticed that (a) an average concentration of about $4 \mu g$ per ml plasma, occurring three minutes after

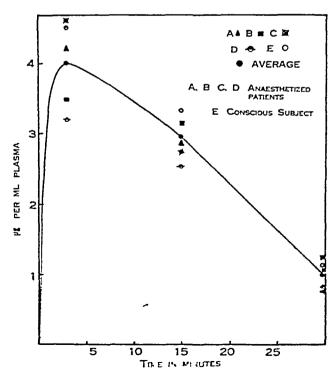


Fig 1—Plasma concentrations of tubocurarine in man after the intravenous administration of 0.2 mg/kg

the injection, seems desirable for the production of full muscular paralysis, providing adequate relaxation for surgical procedures. This corresponds in the conscious subject to the complete classical picture of curarization, (b) fifteen minutes after the injection, when the muscles begin to regain their tone, and in the conscious subject their power, the corresponding average concentration is about $26\,\mu g$ per ml plasma, and (c) half an hour after the injection, when there was apparent recovery from the drug effects, a level of about $1\,\mu g$ per ml plasma was reached

The various volumes of distribution of the drug at the specified intervals are given in Table III

TABLE III

VOLUMES OF DISTRIBUTION OF TUBOCURARINE IN MAN

Dose 0.2 mg/kg 1 v

Time	Mean	Log (mean concen dose (mg /kg))	Volume of
after	concentration		distri-
dose	in plasma		bution
(min)	(mg/)		I /100 kg
3	4 0	1 30	5
15	2 6	1 11	77
30	1 0	0 7	20

In the conscious subject the drug was detected in the saliva in a concentration of 12 μg per ml twenty-one minutes after the injection, and in the CSF in a concentration of 25 μg per ml thirty-three minutes after the injection. About 12 per cent of the dose injected was recovered from this subject's gastric juice

The gastric excretion of tubocurarine was also demonstrated in cats in the following way

In spinal cats, previously starved for 15-18 hours, the stomach was washed with warm saline solution, tied at the cardia, and filled with 80 ml saline through a cannula tied into the pylorus. Adequate artificial ventilation was maintained. The drug was injected in a dose of 0.2 mg/kg intravenously through a cannula connected to the femoral vein. One hour after the injection a sample of saline was withdrawn from the stomach and examined for its tubocurarine content. Two such experiments were performed, in one, the total excretion was equivalent to 19.4 per cent of the dose injected (cat \$2 kg) and in the other (cat \$2 kg) to 14 per cent.

Renal excretion—The tubocurarine equivalents of the hourly samples of urine obtained from the five human subjects are shown in Table IV

TABLE IV , THE RENAL EXCRETION OF TUBOCURARINE IN MAN Dose 02 mg /kg 1 v

Sub-	Dose	Tu	Total excretion				
ject m	mg	1st hr	2nd hr	3rd hr	Next 3 hrs	Total	as % of dose given
A B C D E	14 15 12 15 15	2 82 2 48 2 20 2 6 3 0	1 24 1 82 1 24 2 02 1 4	0 50 0 72 0 62 1 50 1 0	0 0 3 0 0 0	4 56 5 32 4 06 6 12 5 4	32 6 35 5 33 8 40 8 36 0

TABLE V
BLOOD CONCENTRATIONS AND VOLUMES OF DISTRIBUTION
OF TUBOCURARINE IN RABBITS

Dose 0 12 mg /kg 1 v

Rabbit		Tubocurarine concentration mg/l of plasma at times stated after dose				
No	Weight kg	2 min 10 min		15 min		
1 2 3 4 5 6	2 2 4 2 2 5 2 8 2 8	21 20 23 24 22 22	1 2 1 7 1 5 1 7 1 4 1 5	0 8 1 2 1 0 1 1 1 0 0 9		
Mean con	ncentration	2 2	1 5	_ 10		
Log(mean concen dose (mg /kg)		1 262	1 097	0 92		
Volume of distri- bution 1/100 kg		5 4	8 0	12 0		

The fate of tubocurarine in the rabbit

The drug was injected intravenously in a single dose of 0.12 mg/kg body weight. At the 2nd, 10th, and 15th min after the injection blood samples were collected and the plasma separated as usual. Urine samples were usually collected by a sterile catheter at the end of the 2nd, 4th, and 7th hours after the injection. All samples were extracted and examined for their curariform activity. Six rabbits of both sexes were used

In Table V the blood concentrations and volumes of distribution at the stated intervals after the administration of the drug are shown. Fig 2

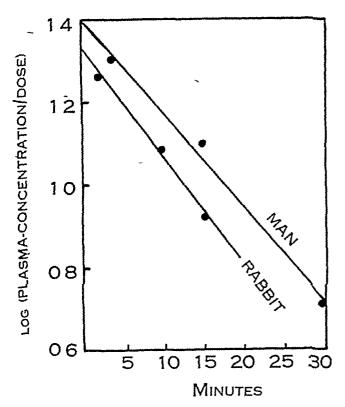


Fig 2—The concentration-dose relationship after intravenous administration of tubocurarine (0 12 mg/kg) in the rabbit and (0 2 mg/kg) in man

represents the relationship between time and the logarithm of the ratio $\left(\frac{\text{mean conc in plasma}}{\text{dose mg/kg}}\right)$ for man and the rabbit. In this Fig. it may be noted that the ordinate corresponding to zero time for man is 1.38 or log 24, the volume of immediate distribution is thus estimated as 100/24 or 4.2 per cent, which is probably about equal to the plasma volume. For the rabbit the volume of immediate distribution calculated in the same way was 4.7 per cent. This is consistent with the finding that the drug does not pass into the blood cells. It disappears from the plasma exponentially, with a halving time of about 13 min.

The average total excretion of the drug in the rabbit's urine was found to be 35 per cent of the dose given, of this about 23 per cent was excreted in the first two hours and 12 per cent in the second two hours. Samples collected at the end of the seventh hour were free from the drug.

Distribution in rabbit's tissues

The distribution of the drug in the rabbit's tissues was examined in two rabbits of different sex and of equal body weight. Ten minutes after the intravenous injection of 0.17 mg kg the animals were killed by stunning and bleeding, and their organs.

removed and extracted Extracts of brain, kidneys, liver, and voluntary muscles were examined, and the results are shown in Table VI

"TABLE VI
TUBOCURARINE-EQUIVALENTS OF RABBIT'S TISSUE
EXTRACTS

Rabbits killed 10 min after the intravenous administration of 1 17 mg/kg

	Voluntary muscle	Brain	Kıdneys	Liver
Tubocurarine equivalent (μg /g)	0 16	0 12	1 6	0 10
	0 15	0 15	2 5	0 13
Total equivalent of organ (µg)	144	1 27	25 6	9 5
	135	1 50	35 0	11 44
Percentage of dose	42	0 37	7 5	2 8
in organ	40	0 44	10 3	3 4
Average % of dose in organ	41	0 41	8 9	3 1

In one rabbit the muscle extract was prepared from neck muscles and in the other rabbit from the thigh muscles. No appreciable difference between the concentrations of the drug in the two extracts was noticed. The calculation of the total tubocurarine-equivalent of voluntary muscles was made on the assumption that they constitute 45 per cent of the total body weight.

Oral administration

Rats of both sexes weighing between 200 and 280 g. were used, and tubocurarine was administered by a stomach tube after the animal had been starved over-night. The results are shown in Table VII

TABLE VII

FATE OF TUBOCURARINE GIVEN BY STOMACH TUBE TO RATS

(No drug detected in faeces)

Dose mg/kg	Number of rats	E ects	Urine % of dose	
10 25	5 5	None seen	Nil "	
30 35 40	2 5 3	Variable paralysis Severe paralysis	0 15 0 1 0 2	
42	, 5	Severe paralysis and death		
45	3	do	-	

From these experiments it became clear that the drug is absorbed from the gastro-intestinal tract. In an attempt to localize the site of absorption from this tract the following experiments were performed.

Group I rats—Each animal in this group was starved over-night and in the morning a median laparotomy incision was made under ether anaesthesia and the duodeno-pyloric junction secured and Then a stomach tube was passed through the mouth, with the animal still under anaesthesia, and tubocurarine injected through the tube into the stomach The abdominal incision was then stitched up quickly and the animal allowed to recover from the anaesthesia The presence of large amounts of the drug (50, 100, and 120 mg/kg body weight) introduced into the stomach in this way was without any obvious effects for a period of two hours, after which the animal was painlessly killed Two animals were given each of the lower doses and four the higher dose The weight of these rats ranged between 200 and 260 g When the animal was killed and the stomach contents examined, practically all the amount introduced was recovered from there, the drug was not absorbed by the gastric mucosa

Group II rats—Each animal was starved overnight and in the morning a median abdominal incision made under ether anaesthesia and the duodeno-pyloric junction secured. A stomach tube was passed through the mouth and manipulated from the abdominal wound into the duodenum. The tube was then kept in position by a loose loop placed around the duodeno-pyloric junction. The drug was introduced into the small intestine by injecting it through the stomach tube. Then the latter was carefully withdrawn while the loose loop was tightened around the duodeno-pyloric junction. The laparotomy incision was then quickly stitched up and the animal allowed to recover from the anaesthesia.

On recovery from anaesthesia, these rats were observed to pass quickly into a typical condition of curare paralysis of variable severity. In six rats weighing between 200 and 250 g when the dose of tubocurarine left inside the intestine was over 3 mg/kg (3 5 mg/kg. in four rats and 4 mg/kg in two rats), paralysis was very severe and progressed to complete respiratory arrest and death in 3-8 min Doses of 2-3 mg per kg invariably produced a certain degree of paralysis of variable severity. starting about 4-6 min after the internal administra-This paralysis extended over a period of tion 15-25 min and was severer when the dose left inside the intestine was 3 mg/kg Four rats weighing between 200 and 260 g were used for each dose level

It was also noticed that the duration of action of the drug was rather short, although absorption started fairly soon. It was suspected that the pancreatic juice might be causing inactivation of the drug. In order to investigate this the pancreatic juice of a cat, prepared by the method recommended by Sherrington (1919), was incubated with tubocurarine at 37° C and the curariform activity of the mixture evaluated at the end of two hours. No loss of the tubocurarine content of the mixture was detected. The pancreatic juice thus does not appear to catalyse the destruction of tubocurarine.

The effect of water duresis

In these experiments the tubocurarine-equivalent in the urine of a group of rats was determined after the intramuscular administration of 03 mg tubocurarine per kg These rats were starved over-night, and on the following day they were injected with the same doses of the drug, just after they had received 50 ml water per kg body weight by a stomach tube, and the tubocurarine equivalent of their urine was again determined Three groups of rats, A, B, and C, each containing three male rats, were used The total weight of group A was 745 g., of B 750 g, and In all groups urine specimens were of C 730 g collected at the end of the fifth hour and the ninth hour after the injection, the second samples were mactive, but the fifth hour samples showed curariform activity When the total tubocurarine-equivalents of the urine were calculated, in each case, before and after the water diuresis, an increase in the total equivalent was noticed to have occurred during the These results are shown in Table water diuresis VIII

TABLE VIII

URINARY EXCRETION OF TUBOCURARINE IN RATS, WITH

AND WITHOUT WATER DIURESIS

Dose 0.3 mg/kg intramuscularly

	DOSC OF ING			
Rats	Urine volume (ml in 5 hr)	Total tubocurarine-equivalent excreted (µg)	% of amount administered	
Group A	5	44 7	20	
Group B	3 8	52 8	23 5	
Group C	4 5	39 4	18	
After 50 m	nl water/kg boo	iy wt by stomac 68 2	20 6	
Group B	15	63	28	
Group C	18	67 8	31	

It was also noticed that although the kidneys seem to be an important organ in the elimination of the drug, renal damage did not prevent full recovery of the animal from the paralytic effects of the drug This was illustrated by a series of experiments on doubly nephrectomized rats, where it was observed that the removal of both kidneys did not seriously affect the reactions of the animals to tubocurarine Such animals were apparently able to cope with paralytic doses of the drug (0 3 mg/kg body weight intramuscularly), and, although the average duration of action of the drug was increased by about 30 per cent, the recovery of the animals by the end of this period was almost complete Hepatectomy (about 75 per cent of the liver) did not appreciably affect the sensitivity of rats to the drug.

TABLE IX
BALANCE SHEET, SHOWING RECOVERY OF TUBOCURARINE
IN MICE

Dose 02 mg/kg 1 v

Time in hours	Wt of mice (g)	Dose per mouse (mg)	Per cent recovery		
			Mice	Excreta	Total
0	20, 20	0 004	92	0	92
	22, 22	0 0044	93	0	93
1	20, 20	0 004	76	0	76
	25, 25	0 005	80	0	80
4	22, 22	0 0044	20	30	50
	25, 25	0 005	10	22	32

A balance sheet for tubocurarine—This was constructed from experiments on mice, in which the drug was injected intravenously in a dose of 0.2 mg/kg. Pairs of male animals weighing between 20 and 25 g were used and the excreta were collected at variable intervals after the injection. The animals were killed and minced, and the tubocurarine equivalent of the extracts of the mince and of the excreta determined. Table IX shows the relationship between the amounts in the mice and in the excreta and the percentage recovery of the dose

Discussion

The various stages of tubocurarine paralysis could be correlated with the concentrations of the drug in the plasma though the concentrations at the neuro-muscular junction must be more intimately related to these effects. The degree of this paralysis varies

widely in different individuals (Gray and Halton, In the conscious human subject no apparent changes in the sensations were observed to follow the injection of tubocurarine, this was also observed by Prescott et al (1946) and by Smith et al (1947), although it has been reported by Whitacre and Fisher (1945) that intocostrin produces general The presence in the CSF of this subject of curariform activity equivalent to 2.5 μg tubocurarine per ml may be of clinical interest. It occurred at a time when the concentration of the drug in the plasma was about 1 µg per ml Everett (1948) has shown that when the drug is brought into direct contact with the central nervous system in a sufficient concentration, it is liable to set up convulsions of central origin The occurrence of violent convulsions after the intravenous administration of tubocurarine in a case of schizophrenia was reported by Morrison (1948), this may have been due to a greater leakage of the drug from the vessels of a pathological central nervous system The drug is excreted by the salivary glands and gastric mucosa The excretion of curarine along these channels was reported by Koch (1870), and von Huber (1922) drew attention to this fact this respect, tubocurarine is behaving in a similar way to some heavy metals and alkaloids, eg, morphine The amounts of tubocurarine excreted this way, however, are insufficient to produce poisoning after reabsorption

The renal excretion of tubocurarine after an intravenous injection is a relatively slow process In hourly samples taken from man it was possible to detect it in the urine three hours, and sometimes four hours, after such an administration might explain the common observation of the anaesthetist that if he has to give a second dose of tubocurarine during a lengthy operation, he usually requires a smaller dose to produce a full effect; some of the previous dose is probably still in the system In the rabbit, as early as ten minutes after the intravenous administration of the drug, the apparent tubocurarine content of the kidneys per gramme of tissue weight was already higher than that of other organs, where the drug seemed to be uniformly distributed

Although the kidneys appeared to be playing an important part in the elimination of the drug, yet the relief from the obvious effects of curarization did not seem to depend entirely upon renal excretion. In rats, although the total removal of both kidneys caused a slight increase in the duration of action of the drug, yet the recovery of the animals by the end of this period was complete. Partial hepatectomy

did not appreciably increase the sensitivity of these animals to tubocurarine. It was also concluded by Rothberger and Winterberg (1905), and later by Polimanti (1914), that the liver plays no part in detoxicating the drug

These findings agree with recent clinical observations by Wall (1947) that renal and hepatic damage do not necessarily constitute a serious contradiction to the clinical use of the drug

There must be some other mechanism by which tubocurarine disappears from the body. The experiments on mice show that the drug is inactivated in the body, since 60 per cent of the dose injected disappeared in 4 hours. The site of this inactivation is unknown. It may perhaps occur in voluntary muscle which was found to contain 40 per cent of the dose in the experiments on rabbits.

It is almost a popular belief that curare 1, ineffective when given by mouth, either because it is not absorbed from the gastro-intestinal tract or because it is destroyed there or because it is excreted as quickly as it is absorbed, so that an effective blood level is not easily reached Bernard (1857) showed that the drug given by mouth to dogs was not destroyed by the gastric juice

Here it was noticed that, within a certain range of dosage, typical paralytic effects were produced when tubocurarine was given by stomach tube to rats, thus indicating absorption. However, the presence of large amounts of the drug in the stomach alone (over 100 mg/kg body weight) was without any obvious effects on the animal, this was probably due to lack of effective absorption from this organ. When the drug was introduced directly into the small intestine, in a much smaller dose (2–3 mg/kg), signs of absorption developed rather rapidly and progressed fatally with a slight increase of this dose

It is possible that with such drugs, producing obvious characteristic signs within a short period after administration, the widely different absorbing properties of these neighbouring mucous membranes could be demonstrated pharmacologically. This may be another instance of the use of tubocurarine as a pharmacological tool

The effects of the drug (2-3 mg/kg) thus absorbed were, however, of short duration, since in 15-25 min the animal recovered from the obvious drug effects. It seemed unlikely that the relatively slow renal excretion could be keeping pace with such a rapid absorption to an extent which would prevent the development of a dangerous blood level. It is possible that the continuation of absorption from the small intestine was limited by a process of precipitation and that the drug may be further

destroyed along its course in the intestines Clement and Pistorio (1928) showed that bile and bile salts could precipitate the alkaloid from curare

The direct administration of the drug into the intestine reduced the size of the effective dose by mouth more than tenfold. It might be possible to imitate this clinically by giving the drug in keratin-coated capsules in the hope of getting desirable effects in spastic paralytic conditions, but probably the limited absorption of the drug from the intestine and the short duration of its action when so absorbed may limit the clinical value of the drug administered that way

SUMMARY -

- 1 The method described by Jalon for estimating tubocurarine by its action on the frog's rectus abdominis was adopted for determining the drug equivalent of tissue extracts and biological fluids. This method was used to follow the fate of the drug in man and animals
- 2 The immediate volume of distribution on intravenous injection corresponds to the plasma volume. The drug does not enter the blood cells. It disappears from the plasma exponentially with a halving time of about 13 min. These conclusions apply both to man and to rabbits.
- 3 About 20-40 per cent of the drug appears in the urine This percentage may be increased by water diuresis Excretion continues for several hours even when the paralysis only lasts about half an hour
- 4 The main route of disappearance of the drug from the body does not depend on the kidneys By extracting whole mice it was shown that about 60 per cent of the dose was inactivated in the body within four hours. The liver is probably not the main site of inactivation. It is possible that inactivation occurs in voluntary muscles, which were found to contain 40 per cent of the dose in an experiment on rabbits.
- 5 The effective dose by oral administration in rats is about 100 times the effective dose by intramuscular administration. Absorption occurs in the small intestine, but not in the stomach. On intravenous injection, appreciable quantities (12–19 per cent of the dose) may be excreted into the stomach.

It is a pleasure to express my indebtedness to Professor J H Gaddum for suggesting this problem, and for continued guidance and stimulating interest I am deeply grateful to Dr M Vogt for the valuable help and criticism she has given throughout this work, and to Dr J Gillies and Dr H W Griffith of the Anaesthetic Department, Royal Infirmary, for the facilities they

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THE PRESSOR AND DEPRESSOR EFFECTS OF CERTAIN SYMPATHOMIMETIC AMINES

BY

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Amphetamine and its dextro-isomer are widely used in obesity, in patients who are often liable to suffer from hypertension. Methyl-amphetamine (methedrine) and related substances are also frequently given in repeated doses for their blood-pressure raising effect. As the pharmacological action of these agents becomes weaker on repeated administration, it appeared of interest to reinvestigate and compare their effects.

According to Beyer (1946), the diversity of the response to these agents is not adequately accounted for by Gaddum and Kwiatowski's (1938) theory, which explains the action of ephedrine as being due to its power to inhibit the destruction of adrenaline by amine oxidase. The pressor effects of aromatic alkylamines under various experimental conditions were therefore compared with the action of adrenaline and with each other.

METHODS

Cats were anaesthetized with pentobarbitone intravenously or intraperitoneally, or with ether. Blood pressure was recorded by a mercury manometer connected with a cannula in the carotid artery. All drugs were injected into the external jugular vein. Respiration was recorded by means of a tambour attached to the thoracic or abdominal wall.

RESULTS

A slight increase of blood pressure after an injection of ephedrine, racemic or d-amphetamine sulphate, or methedrine could be observed with doses of 0 002 mg /kg and upwards. The rise was usually maximal with 1 mg /kg. In different animals the response varied greatly both in intensity and duration. After 0.2 mg /kg dexedrine, the pressor effect lasted for eight minutes in one animal, but it took up to fifty minutes in other cats before the original level was reached.

This individual variation was also present in spinal animals. Within the range of pressor responses no definite quantitative difference was

observed between the various amines which were tested

Curtis (1929), in his studies on ephedrine and related substances, has pointed out that the diminished response to repeated injections makes it impossible to compare quantitatively the effect of these substances in the same animal. It was found, however, that some comparison could be made when small amounts of two agents were injected alternately at short intervals. When using this method for racemic amphetamine and its *dextro*-isomer, we observed a gradually diminishing pressor effect as if the same substance had been injected all the time (Fig. 1)



Fig 1—Blood pressure effect of alternate doses of 0 1 mg/kg racemic (A and C) and dextro- (B and D) amphetamine sulphate in a spinal cat Time minutes

The small effect of repeated doses of the amines was followed, when injections were continued, by a fall of blood pressure, except with ephedrine, which never caused such an inversion. The amount of the sympathomimetic amine required for the production of such tolerance again varied individually. Usually 1 to 3 mg/kg were necessary, given either in one or two doses or by injecting smaller amounts repeatedly (Fig. 2). After some hours tolerance to a fresh injection disappeared and

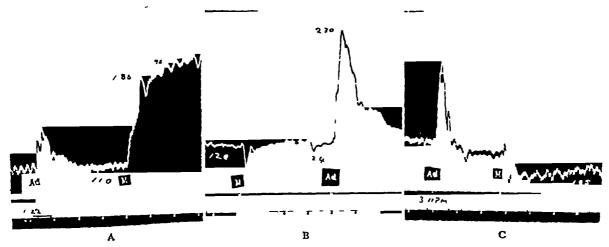


Fig 2—Blood pressure effect of repeated doses of 1 mg/kg methedrine (M) in a cat! (pentobarbitone)
The pressor effect is marked in A, but reversed in B and C almost 4 hours later, 0.5 ml
adrenaline 1/100,000 (Ad) is pressor throughout Time minutes

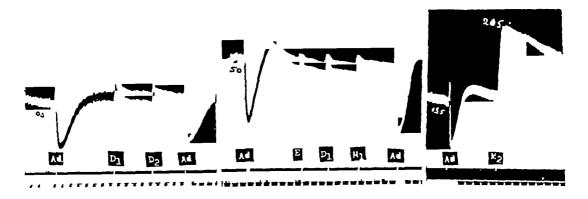


Fig. 3—Pressor effect of dextro-amphetamine sulphate, ephedrine, and methedrine, and depressor effect of adrenaline in a cat (ether). Knee jerk abolished throughout. Time minutes Ad = 0.5 ml adrenaline $1/100,000 D_1 = 0.0017 \text{ mg/kg}$ d-amphetamine sulphate $D_2 = 0.00085 \text{ mg/kg}$ d-amphetamine sulphate $D_2 = 0.0018 \text{ mg/kg}$ d-amphetamine sulphate $D_3 = 0.0018 \text{ mg/kg}$ methedrine $D_4 = 0.0018 \text{ mg/kg}$ methedrine

a pressor response occurred again. When large doses of the drugs had been given, however, an inverted response was observed even when several hours had been allowed to elapse after the first series of injections. Atropine had no influence on the decreased or inverted responses

In producing this tolerance to repeated doses the various amines were interchangeable, for example, ephedrine would prevent a rise of blood pressure in response to a later injection of amphetamine or methedrine and vice versa. The inverted response after repeated injections was not influenced by atropine, and it was obtained in the decapitated cat

While the blood pressure effects of ephedrine, racemic amphetamine, dexedrine, and methedrine were very much alike, adrenaline acted differently under our experimental conditions. Under pentobarbitone anaesthesia its pressor action persisted

even when large amounts of other amines had been given (Fig. 2)

When, under ether anaesthesia, the response to adrenaline became depressor the other amines retained their pressor effect for their initial doses. This applied both to small and large amounts of the substances (Fig. 3)

The pulse rate in the anaesthetized animals remained substantially unchanged during these depressor responses

DISCUSSION

Both aliphatic amines and aromatic alkylamines have a decreased blood pressure effect when injected repeatedly (tachyphylaxis). Tainter (1929) has described this for ephedrine, and Ahlquist (1943) has shown it for various aliphatic substances with a sympathonimetic action. Elmes and Jefferson (1942) injected ephedrine into the anaesthetized cat

at hourly intervals and found the pressor effect to decrease gradually; Burn (1946) observed a similar effect with methedrine Detrick et al (1937), confirming the observation for amphetamine, found in dogs great variability of the pressor response Pinkston et al (1939) and Pinkston and Pinkston (1939) state that after an initial dose of 1 mg/kg the magnitude of the response varied directly with the dosage up to 6 to 8 mg/kg Clinical studies by Myerson et al (1936) and Reifenstein and Davidoff (1938) stress the irregularity of pressor responses to amphetamine in man

Our experiments show that in the anaesthetized cat 0 002 mg/kg of these amines will usually produce a very slight rise of blood pressure. The pressor effect becomes sustained after 0 2 to 0 5 mg/kg and maximal with 1 mg/kg. It may last for almost an hour after a large dose in one cat and for a few minutes only in another. We did not find that the depth of anaesthesia had much influence on this variability.

The strength of pressor action of various agents can be compared in the same animal when small doses are given. Though tachyphylaxis develops, one may find a larger or smaller pressor action when the respective substances are injected at short intervals of one another, but an exact comparison could not be made. Alles (1939) and Hauschild 1940) found that racemic amphetamine and its dextro-isomer had the same pressor effect, while Swanson et al. (1943) considered the laevo-isomer to be more powerful in its pressor action. Our own observations did not show any appreciable difference between the various forms of amphetamine.

That the differences of blood-pressure effects between various animals are not due to a varied sensitivity of the central nervous system and, particularly, that they are not secondary to the action on the respiratory centre was shown in the decapitated cat. Here, as well as when atropine had been given, the same individual differences were observed and tachyphylaxis developed in the same way as in the intact animal

Several stages could be observed as the animals became gradually saturated with the drugs

- 1 The pressor effect became smaller and was eventually absent as if an inert substance had been injected
- 2 A momentary drop of blood pressure was immediately followed by a short elevation resulting in a biphasic tracing
- 3 The blood pressure fell and returned to its original level sometimes quickly and sometimes very slowly

However, ephedrine never led to a drop in blood pressure, though the diminution and eventual disappearance of the pressor effect developed as readily as in experiments with amphetamine and methedrine

The depressor effect was explained by Ahlquist (1943, 1944) and Jackson (1944) as being due to myocardial depression. In flow meter studies on the dog's hind leg, Ahlquist (1945) found that the depressor response was partly due to peripheral vaso-dilatation, and that it was similar to the dilatation after adrenaline. From our observations of the pulse rate during the depressor phase, there was no evidence of a direct effect on the heart. As the depression is not influenced by atropine and as it is also present in the spinal cat, it appears to be due to a vaso-dilator action in the periphery

The pressor action of aliphatic amines can be blocked by repeating doses or by ephedrine (Ahlquist, 1945) The same applies to the sympathomimetic effect of aromatic alkyl-amines Hence, once ephedrine, amphetamine, dexedrine, or methedrine have been given in fair doses, further injections will not cause a rise of blood pressure and may, apart from ephedrine, even produce a depressor effect which lasts for a considerable time

The effect of the amines tested was in our experiments quite different from that of adrenaline. Under pentobarbitone or in the spinal cat the adrenaline response remained pressor. Likewise when under ether anaesthesia adrenaline acted as a depressor, ephedrine, amphetamine, dexedrine and methedrine had their usual pressor effect until tachyphylaxis developed. These differences between adrenaline and the other sympathomimetic amines when given to the same animal at short intervals of each other made it clear that under the given experimental conditions their mechanisms of action are not identical.

It appears difficult to reconcile these results with the theory of Gaddum and Kwiatowski (1938) according to which adrenaline is the substance through which ephedrine acts. If this were true the action of adrenaline and the other sympathomimetic amines would be more or less alike. Tainter (1929, 1933), Aström (1948), and others have shown differences in the effect of ephedrine and other pressor amines.

Tainter (1929) concluded that ephedrine and adrenaline did not have the same seat of pressor action. The opposite responses to the group of sympathomimetic amines on the one hand and to adrenaline on the other shown in our experiments make it likely that their modes of action are different.

Bever (1946) suggested that the action of sympathomimetic amines might be explained by their effect on the formation of adenosinetriphosphate as influenced by various breakdown products of metabolism and by their influence on the efficiency with which energy is used This would allow more flexibility than would be permissible if the action of all sympathomimetic amines were mediated through one common agent, adrenaline It would allow for differences between the effect of adrenaline and the other amines described in this paper. The observation by Govier et al (1945) that certain concentrations of amines lead to an increase of oxidations while larger amounts produce an inhibition of oxygen uptake might help to explain their diminishing pressor effect and the eventual reversal to a depressor action

We are indebted to Messrs Menley and James, Ltd for a supply of devedrine

SUMMARY

- 1 In the anaesthetized and in the spinal cat racemic amphetamine and its d-isomer are equally potent in their effect on the blood pressure
- 2 Ephedrine differs from amphetamine, its isomers, and methedrine in that on repeated doses it does not cause a fall of blood pressure
- 3 As the various amines are interchangeable in blocking the pressor effect an injection of methedrine may result in a drop of blood pressure when either ephedrine, amphetamine, or methedrine itself has been given beforehand in fair dosage

- 4 None of these effects is influenced by atropine
- 5 The pressor effect of adrenaline persists independently of the changes in the action of the other amines. When, under ether, adrenaline is depressor the other amines have a pressor effect. Under pentobarbitone and in the spinal cat adrenaline still raises the blood pressure when, through repeated injections, ephedrine has lost its effect and amphetamine and methedrine have become depressor agents.

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EFFECT OF REDUCTION OF POTASSIUM ON THE ACTION OF ACETYLCHOLINE ON RABBIT AURICLES

ΒY

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Spadolini and Domini (1940) were the first to show that minute doses of acetylcholine may stimulate the isolated heart of the guinea-pig and they suggested that this action might be due to a release of adrenaline in cardiac tissue Hoffmann. Hoffmann, Middleton, and Talesnik (1945) and Haney and Lindgren (1946) have shown that acetylcholine may have a stimulant effect on the heart of the cat or dog after the administration of atropine, and McDowall (1946) has further shown that small doses of acetylcholine may stimulate the cat heart untreated with atropine (1946) and de Elío (1947) have shown that acetylcholine reduces the refractory period of the isolated driven auricle of the rabbit, and Wedd and Blair (1946) have demonstrated the direct nature of this effect on nerve-free tissue from the ventricle of the turtle Recently Burn and Vane (1949) and Bulbring and Burn (1949) have reviewed the literature concerning this subject and as a result of studies of the effect of proguanil and of fatigue on the isolated rabbit auricle have suggested that acetylcholine may play a dual role in the heart Synthesis of acetylcholine proceeds in the contracting auricle, addition of acetylcholine to produce a concentration above the normal amount present in the tissue causes inhibition of contraction the muscle is depressed addition of acetylcholine from without may bring the concentration up to the threshold for normal functioning and bring about stimulation of the heart

The relationship between the concentration of potassium ions in a tissue and the effects thereon of administered acetylcholine is a close one. In the perfused frog heart increase or decrease of potassium in the perfusing fluid (with appropriate control of the pH, saline content, temperature, etc.) reduces the activity of the heart, makes the rhythm irregular and modifies the response to acetylcholine. Similarly increase or decrease of K modifies the contraction of the isolated rabbit auricle.

EXPERIMENTAL

It was decided to test the effect of modification of the ionic content of potassium on the response of the rabbit auricle to added acetylcholine. The beat of the isolated rabbit auricle was recorded as usual at 27° C in powerfully oxygenated Locke solution with double the normal concentration of glucose. At suitable intervals this fluid was replaced by a modified Locke solution containing half or one and a half the normal amount of potassium. The pH was measured and adjusted if necessary by modifying the bicarbonate content to give pH 8.2

The addition of 0.25 μ g acetylcholine in a 75 ml bath may produce a preliminary stimulation of the auricle of small extent (Fig 1 a and b) followed by inhibition. In some specimens this inhibition is not so transient (Fig 1 c) but frequently there is either no effect from addition of such quantities

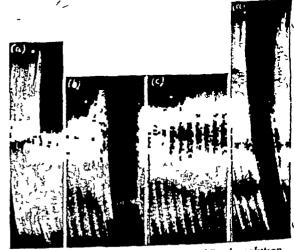


Fig 1—Isolated rabbit auricle in normal Locke solution at 27° C (a) and (b) show transient stimulation after adding 0 25 μg acetylcholine to the 75 ml bath (c) shows a longer stimulation after the same amount of acetylcholine (d) shows inhibition after 1 0 μg

of acetylcholine or a pure inhibition. The tendency to preliminary transient stimulation develops as the preparation gets older and has been seen in nine out of 47 auricles after two hours beating In four specimens it was apparent after one hour, it was also seen in two auricles (excluded from the series) which would not beat until they had been treated with 100 μ g acetylcholine followed by washing after a few minutes Addition of larger quantities of acetylcholine (1 µg or more) causes inhibition of the activity of these preparations, which may be interrupted by a few beats more powerful than normal (Fig 1 d) Increase of the potassium in the nutrient fluid abolishes this early stimulation with small quantities of acetylcholine, which now have no effect on the heart, or inhibit it

If the amount of K is reduced the augmentor response to added acetylcholine is enhanced in those preparations which show this effect. Quantities of acetylcholine which, in normal Locke solution, previously or subsequently caused a moderate inhibition of the contraction for a prolonged period might now cause a preliminary stimulation followed by a sharp but transient inhibition, and frequently a prolonged phase of increased activity (Fig. 2 a). These effects can be repeated with considerable regularity in those preparations which show the phenomenon (37 out of 45 tests) and are in contrast with the usual action of acetylcholine on the isolated auricle in normal nutrient fluid (Fig. 2 c and d)

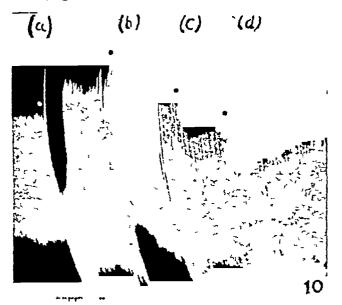


Fig 2—Isolated rabbit auricle (a) and (b) show the effect of adding 10 µg and 50 µg of acetylcholine respectively, without any wash between, to Locke solution containing half the usual amount of potassium (c) and (d) as in (a) and (b) but in normal Locke solution For description see text

DISCUSSION

If isolation of the auricular tissue in Locke solution brings about a state of fatigue and some loss of activity in the muscle, and thus a diminution of synthesis of acetylcholine, addition of a small quantity of acetylcholine will bring the amount of this substance up to the optimal value and cause stimulation of the contraction sequence of events is seen in isolated auricles which develop the power of responding to small amounts of added acetylcholine by increased activity, after the preparation has been up for Many specimens never show this some time property but the same or a similar phenomenon is shown by auricles which have ceased to beat from fatigue and which can be restarted by addition of larger amounts of acetylcholine, with or without washing (Bulbring and Burn, 1949), and by auricles which are inhibited from the beginning, perhaps as a result of rough handling, and which begin to beat after the addition of relatively large doses of acetylcholine, followed by washing Reducing the activity of the muscle by increasing the potassium content does not bring about a state of affairs in which acetylcholine causes stimulation Reduction of the potassium content on the other hand not only modifies the activity of the muscle but also alters the response to acetylcholine, so that amounts of this substance which previously caused inhibition now cause stimulation Rapid alterations of the response between stimulation and depression may be seen (Fig 2 a) at certain concentration levels of acetylcholine, whereas at higher levels pure depression is observed (Fig 2 b) It may be that an optimum level of potassium is necessary for optimal synthesis of acetylcholine, or for the inhibitory response of the muscle to it. The nature of the response to added acetylcholine would then depend on the condition of the muscle, especially as to its potassium content, and on the amounts of acetylcholine produced in the muscle and applied to it externally, as Burn and Vane suggest Potassium would appear to play as intimate a role in the positive as in the negative response of cardiac tissue to acetylcholine According to Jequier. Plotka, and Petergalvi (1948) the connexion between K and acetylcholine synthesis may lie in the relation between K and degradation of glucose

SUMMARY

Low concentrations of acetylcholine (3×10^{-9}) may cause a transient stimulation of the isolated rabbit auricle contracting in Locke solution Higher concentrations inhibit it.

Relative lack of potassium causes doses of acetylcholine, which previously caused a slight transient stimulation, to cause a much larger preliminary stimulation, and some doses which previously inhibited, to stimulate

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ISOPROPYLNORADRENALINE INHALATION AND MUCOUS MEMBRANES

BY

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Inhalation of aerosols is now widely used in the treatment of bronchial asthma. Numerous substances are employed, of which adrenaline and its related compounds are the most powerful spasmolytics. The relief afforded is so striking (though often temporary) that numerous preparations and nebulizers are bought by the public, very often without medical advice or supervision.

In these circumstances it appears important to know whether inhalation of these compounds may be harmful to the mucous membranes of the air passages Galgiani et al (1939) found that inhalation of 1 per cent adrenaline in rabbits and cats caused damage to the mucous membranes of trachea and bronchi Their results are partly supported by earlier findings of Fox (1931), who sprayed the nasal membranes of rabbits with 1/1000 adrenaline and found a mucopurulent discharge

Very recently *iso*propyl*nor*adrenaline* (aleudrine) has become available. This substance is easily absorbed from the mucous membranes of mouth, pharynx, and the other air passages, and its spasmolytic action appears to be very strong (Herxheimer, 1948) and more powerful than that of adrenaline (Konzett, 1940). Our paper does not deal with the therapeutic merits of *iso*propyl*nor*adrenaline, but as its widespread use can be anticipated it seemed of importance to investigate whether such damage as has been stated to occur after the use of adrenaline would be caused also by this substance

METHOD

Twelve rabbits were used They inhaled an aerosol which was produced by connecting one of the commercial glass inhalers to a compressed air cylinder. A steady pressure was maintained from this cylinder, which caused a fine aerosol cloud to flow continuously from the nozzle of the inhaler. Over this nozzle a closely fitting plastic

mask was fixed which was pressed over the rabbit's head, the aerosol jet issuing from the nozzle was directed straight against mouth and nostrils of the rabbit and filled the mask, escaping at its upper end. Mouth and nose of the rabbit were thus completely and continuously surrounded by the aerosol. That it actually breathed the aerosol could be seen from the fact that the vapour escaped from the mask in the breathing rhythm of the animal. When the animal breathed out the cloud increased strongly, and decreased during inspiration.

The concentration of isopropylnoradrenaline used by one of us (H H) for treatment of asthma is usually 1 per cent, if it is nebulized by pressing the rubber ball of a hand inhaler If a mechanical device, like an airpressure pump or compressed air cylinder, is used, as little as 0 25 per cent is effective, as much more inhalant is breathed than with a hand inhaler. For this reason we started our experiments with @ 0 25 per cent solution, nebulized by compressed air, which was inhaled for 10 minutes every day The twelve rabbits were divided into three groups of four One group inhaled pure aleudrine, one group aleudrine to which 0.2 per cent sodium metabisulphite had been added as a stabilizer, and the third group inhaled physiological saline. In each group one drop of glycerine was added to 2-3 cc of the solution to delay evaporation and to make the aerosol easily visible

All the rabbits were treated in this way for 30 days, then one rabbit of each group was killed by a blow on the head. The remainder continued as before for a further 5 days. Then another rabbit of each group was killed. The remaining rabbits were treated with 0.5 per cent aleudrine (or saline) for 22 days. Then a third rabbit of each group was killed. Only one rabbit of each group now remained. They were subjected for 16 days to 1 per cent aleudrine aerosol except the rabbit in the control (saline) group.

When the rabbits were killed, the trachea was quickly excised, opened longitudinally, put in a bath of Ringer-Locke at 39°C, and pinned without stretching on to a cork. A minute drop of india ink was then dropped on to the mucous membrane half an inch below the cricoid cartilage and its movement towards the larynx (due to the action of the cilia) was watched. The speed of the movement over 0.5 cm was measured with a stop-

This substance was called "isopropyladrenaline" by Konzett (19-0), but since it does not contain the N-methyl group of adrenaline it should be called isopropylroradrenaline—Editors

watch This method of estimating the action of the cilia described by Hill (1928) did not prove very reliable Sometimes movement was very strong in one part of the trachea but not in another, sometimes it was so weak that the india ink travelled only 2 or 3 mm. In many cases, however, a speed of about 1 cm per 50-70 sec was seen, and there were no differences between the Weak movement was observed also in three groups the control group

Trachea and lungs were then fixed in 10 per cent formol-saline and prepared for histological examination, after embedding in paraffin wax Sections were stained with Ehrlich's Acid Haematoxylin and Eosin, with Heidenhain's Iron Haematoxylin, and with Mayer's

mucicarmine stain

RESULTS

The result of the experiments with ciliary movement has already been mentioned. All the rabbits showed steady gain in weight throughout the experimental period Histological examination showed no difference between the three groups The ciliated epithelium was normal. There was no destruction or metaplasia of ciliated epithelium in trachea or bronchi and no polymorphonuclear infiltration of epithelium or submucosa The goblet cells, stained by mucicarmine, were found in approximately similar density in all three groups As compared with three rabbits not exposed to the inhaler, mucous activity in the trachea of the experimental animals was slightly increased

DISCUSSION

There is a striking contrast between our negative results and the positive results of Galgiani et al with adrenaline, applied twice daily by pressing a hand inhaler 10 times They used therefore an amount of inhalant considerably smaller than that During one period of used in our experiments

inhalation (10 squeezes) they removed 8 mg of fluid from the inhaler, whereas in our experiments of 10 minutes duration between 600 and 800 mg were aerolized, the variations being caused by the slight difference in the syphon of the inhaler and of the pressure used This does not mean, of course, that this amount has been inhaled by the animals Only a small fraction of it can have been inhaled and still less absorbed

Of Galgiani's animals (rabbits and cats) exposed to the vapour, 4 died prematurely after 6 to 34 days Two of these showed inflammatory, changes in the respiratory tract Another animal died on the 117th day of pneumonia and empyema remaining animals were killed after varying periods of time Two of them showed loss of cilia and three showed mucopus in the bronchi without histological changes Of the 4 control animals two showed mucopus in the bronchi and a third died with evidence of bronchopneumonia and bronchitis It must therefore remain doubtful whether this result has sufficient weight to prove a harmful action of adrenaline or of the sodium bisulphite which Galgiani et al used as stabilizer In our experiments, sodium metabisulphite did not produce any changes

Our experiments show that aleudrine in therapeutic concentrations is not harmful to the mucosa Whether adrenaline produces harmful of rabbits results remains an open question

We are indebted to Mr T West for assistance in handling the animals and for the histological preparations

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THE AMOUNT OF NICOTINE ABSORBED IN SMOKING

BY

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The work which has been carried out in this laboratory on the effect of smoking during diuresis (Burn, Truelove, and Burn, 1945, Walker, 1949) has made it necessary to determine the amount of nicotine absorbed in smoking cigarettes estimates have been published by different workers These estimates have been made by chemical methods, and the figures vary Bogen (1929) stated that the amount was between 0 2 mg and 6 5 mg per cigarette, according to the rate at which the cigarette was smoked Schnedorf and Ivy (1939) also give the figure 0.2 mg, but Sollmann (1948) states that under average conditions the amount of nicotine entering the mouth from a cigarette ranges from 18 to 85 mg, and the amount absorbed ranges from 14 mg to 33 mg

Storm van Leeuwen (1918) estimated the amount of nicotine in cigar smoke, using the blood pressure of the spinal cat. He obtained figures from 1 6 to 4 25 mg per g of cigar or 8 0 to 20 mg' for an average cigar

It is true that cigarettes can be smoked at very different rates, and it is likely that the amount of nicotine entering the mouth will vary considerably according to the rate. In practice, however, cigarettes are smoked at a rate which is about the same for the majority of individuals, and it should be possible to determine the nicotine within narrower limits than those just given

The work described in this paper consists of two independent investigations, one carried out by H W L in 1935, and the other by C B W P in 1948, each using the same general method. Since the results of the two investigations are very similar, they are probably a better guide to the amount of nicotine entering the mouth in smoking than are the results given in earlier work. The nicotine was estimated biologically on the blood pressure of the spinal cat

Ordinary brands of cigarettes were used in both investigations, and a few estimations of cigar and pipe tobacco were also made by C B W P

METHOD

The cigarettes, cigars, or pipe were fixed in a holder attached to one side of a long glass U-tube, each arm of which was 50 cm long and of internal diameter 1 cm The tube was immersed in a large jar of cold running water, and the other arm was attached to a 500 c c filter flask, which in turn was attached to a weak suction pump A T-piece was inserted between the flask and the pump, and when the arm of the T-piece was open, the pump sucked air in through this open arm, when the arm of the T-piece was closed, air was sucked in through the U-tube A weighed cigarette was inserted in the holder, and when the arm of the T-piece was closed it was easy to light the cigarette. When the U-tube was full of smoke the side arm was opened and suction The cigarette continued to burn gently in the air as it would if a person were holding it Aspirations were then continued to simulate as nearly as possible the smoking of a cigarette, that is to say taking about 10 minutes to smoke the cigarette, so as to leave a stub of about 1 cm The smoke condensed for the most part in the U-tube, any small excess being trapped in the filter flask

To prepare each extract of cigarette smoke, 5 cigarettes (about 5 g of tobacco) were smoked in succession in the apparatus. An extract of cigar smoke was obtained from one cigar (average weight 5-6 g), and pipe tobacco smoke from 6 g tobacco. The time taken for the smoking was suitably adjusted

The U-tube and flask were washed out with N/10 hydrochloric acid to dissolve the deposit, and the extract was filtered It was then made alkaline with KOH and extracted with ether The ether extract was poured into an evaporating basin and left overnight deposit was dissolved in 1 per cent tartaric acid (a few drops of alcohol assisted the process) filtered and estimated The estimations were made by comparing the rise of blood pressure of the spinal cat obtained after injecting doses of the extract with the rise obtained after injecting known doses of nicotine acid The dose required and the time interval tartrate between injections varied in different cats. A dose of nicotine acid tartrate between 0 5-1 0 mg was usually suitable, and a time interval of 5-7 min was usually sufficient to avoid a decrease in sensitivity

This was the method used by H W L, and the method of C B W P was essentially the same. There were two modifications in the later work. Firstly, a set number of aspirations, namely 12, were made in ten minutes for each cigarette, and, secondly, the extract after being made alkaline was extracted five times with ether. It is possible that a more thorough extraction with ether accounts for the slightly higher figures obtained by C B W P.

RESULTS.

1 Obtained by H W L in 1935

A Cigarettes

Each cigarette weighed approx 1 g

Brand	Number of cigarettes used to prepare extract	Amount of nicotine (base) per cigarette
Players Wills Gold Flake	5 10 10	0 66 mg 0 75 mg 0 7 mg

B Cigarette tobacco Wills Gold Flake, 0 6 mg per g tobacco

C Cigars

2 Marcella cigars, 3 8 mg per cigar (each approx 5 g)

2 Obtained by C B W P in 1948

A Cigarettes

Brand	Number of cigarettes used to prepare extract	Amount of nicotine (base) mg per cigarette
Players ,,	5 5 5 5 5	0 83 0 8 0 94 1 00 0 8
State Express 555	5 5 5 5	0 72 1 00 0 98 1.20
	Mean	0 92

B Cigars

- (a) 3 6 mg per cigar (weight about 5 g)
- (b) 6 26 mg per cigar
- (c) 50 mg per cigar
- (d) 79 mg per cigar
- (c) 50 mg per cigar

C Pipe tobacco

Four extracts of 6 g each pipe tobacco were estimated. The results were

(a) 2 mg ni	cotine	per	g to	bacc	0
(b) 2.75 mg	,,	,,	,,	,,	
(c) 3 0 mg	,,	**	,,	,,	
(d) 3 0 mg	"	,,	,,	وو	
Mean = 2.69 mg				••	

DISCUSSION AND SUMMARY

The results show that in two independent investigations made at a time interval of 13 years the amount of nicotine found to be deposited in the smoke from well-known brands of cigarettes was of the same order. The war period has certainly not led to any diminution in the nicotine content. If all the results are considered, a figure between 0.6 mg and 1.0 mg per cigarette or per gramme tobacco is obtained. The cigars yielded between 3.6 mg and 7.9 mg per cigar, or 0.7 mg -1.6 mg per g tobacco. The pipe tobacco yielded between 2 and 3 mg nicotine per g tobacco. This figure for pipe tobacco is higher than the figures for either cigarettes or cigars.

These figures give the amount of nicotine entering the mouth_of the smoker The proportion of nicotine absorbed into the circulation depends on the extent to which the smoker inhales, for mcotine in the smoke entering the alveoli is mostly absorbed Absorption must also occur to some extent in the mucous membranes of the mouth, nose, and throat, for smoking without conscious inhaling raises the pulse rate and the blood pressure Burn, Truelove, and Burn (1945) describe an experiment in which the same individual drank 1 litre of water on two The diuresis was then recorded by occasions collecting urine at 15 min intervals The antidiuretic effect of smoking 1 cigarette was of shorter duration than that produced by the intravenous injection of 0.5 mg nicotine (base) Thus, although the smoker inhaled, less than 0.5 mg nicotine (base) was absorbed into the circulation from one cigarette

It can, therefore, be concluded that, while the amount of nicotine from one cigarette entering the mouth is about 0.9 mg, the amount absorbed is about 0.4 mg or less

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EFFECTS OF SOME *ISO*THIOUREA AND GUANIDINE SALTS ON VARIOUS PREPARATIONS OF SMOOTH AND STRIPED MUSCLE

BY

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(Received July 4 1949)

In seeking pressor agents of new types, Smirk (1941) was led to test the fairly well known organic chemical methyl isothiourea sulphate He found it capable of producing large persistent rises of blood pressure in anaesthetized animals ding to McGeorge, Sherif, and Smirk (1942), the rises of blood pressure are brought about mainly if not entirely through a direct action of methyl isothiourea on blood vessels, and this action is not sympathomimetic They state that the pressor action was hardly affected by the anti-adrenaline compounds, ergotoxine and F933 Methyl 1sothiourea, unlike adrenaline, tended to increase the tone of isolated muscle strips from rabbit intestine, bladder, and uterus

Certain of its chemical relatives have been found to behave similarly the pharmacological properties of methyl isothiourea are reproduced with most fidelity by amidine derivatives like ethyl isourea and guanidine salts which ionize freely to yield cations of small size (Fastier and Smirk, 1943 and 1947, Fastier, 1948) It would appear that pressor analogues of methyl isothiourea need have nothing more in common with it structurally than the amidine group

$$\left(-C\left(\begin{array}{c}NH-\\NH-\end{array}\right)^{+}\right)$$

Now histamine contains this group Moreover, it is known that histamine too causes the contraction of most kinds of smooth muscle irrespective of the innervation. The possibility that amidine derivatives like methyl isothiourea and guanidine produce some of their more characteristic pharmacological effects through simulating actions of histamine has therefore appeared worthy of investigation.

Other experiments reported below were suggested by a much older idea—one which goes

drugs as "Priscol' and d-tubocurarine chloride
Isolated strips of rabbit or guinea-pig ileum and
uterine horns of non-pregnant rats were set up in an
ordinary organ bath of 100 ml capacity. The RingerTyrode solution in the bath was aerated with oxygen
containing 5 per cent carbon dioxide.

The bronchiole preparation employed was that of Konzett and Rössler (1940) Female guinea-pigs of 500-600 g weight were deeply anaesthetized by injecting urethane intraperitoneally Cannulae were inserted into a jugular vein and the trachea The lungs were insufflated with a small Starling respiration pump

back to Fühner (1908), who compared the action of guanidine on striped muscle to that of a univalent alkalı metal cation He pointed out that the effects of both are antagonized by such divalent cations as calcium Similar observations on the interaction of calcium and guanidine have been published by a number of workers (Major and Stephenson, 1924, Ochoa, 1928, Minot, 1931, Burns and Secker, 1935, Harvey, 1940) Minot, Dodd, and Riven (1938) express what is probably a widely held view when they write "It is possible that guanidine exerts its action on muscles through changes in the effect of inorganic salts" For this reason, the effect of changes in calcium or in potassium ion concentration on the response to typical amidine derivatives has also been studied

METHODS

Rabbits' ears were perfused at room temperature with Ringer-Locke solution kept at a constant head of pressure by means of a Mariotte bottle (Kravkov-Pissemski preparation). The venous outflow was measured by the method of Stephenson (1948). Amidine derivatives were injected as salts in neutral solution. The isothioureas tested were some of those specially synthesized for this series of investigations. Samples of guanidine hydrochloride, methylguanidine sulphate, and asym-dimethylguanidine sulphate were obtained from commercial sources, as were also such drugs as "Priscol" and d-tubocurarine chloride

at each stroke of which the excess air from the pump was driven into a float recorder. When the bronchioles were constricted the average level of the marker was raised (see Fig. 3)

Rectus abdominis muscle was taken from winter frogs (Rana temporaria) Each piece was suspended in a 5 ml bath through which air was bubbled slowly Isotonic contractions were recorded with a gimbal lever. The Ringer solution used contained 0.7 g NaCl, 0.014 g KCl, 0.012 g CaCl, and 0.02 g NaHCO, per 100 ml. Acetylcholine was added in known concentration (usually a 1 in 2,000,000 solution) replacing the ordinary Ringer solution of the bath for a period of 90 seconds. The muscle strip was washed two or three times after each dose of acetylcholine. The interval between doses was kept at 5 minutes.

The rat diaphragm preparation was set up as specified by Bülbring (1946) At first the bath temperature was kept at 39° C, but later a temperature of 28° C was chosen as more suitable. Drug solutions were added directly to the bath. A square wave 'stimulator was employed, its construction permitted the amplitude duration, and frequency of the shocks to be varied independently. The nerve was stimulated in the fluid from platinum contacts.

Experiments on cat gastrocnemius muscle were performed on animals under chloralose anaesthesia. The muscle was stimulated via the sciatic nerve with condenser shocks strong enough to give a maximal response. Contractions were recorded by attaching a stout wire from an isometric lever which wrote on a slowly moving drum to the lower end of the tendo Achillis. The central attachment of the muscle had previously been fixed firmly in position by clamping a steel rod driven through the condyles of the left femur. Drug solutions were forced into the left iliac artery through a cannula whose tip lav just below the bifurcation of the aorta.

RESULTS

Effects on smooth muscle preparations Perfused rabbit ear

Methyl isothiourea was tested on eighteen perfused ears. A dose of 0.1 ml of an M/5,000 solution brought about definite vasoconstriction (Fig. 1b) in six of eight experiments. Strong vasoconstriction was caused in all of seventeen ears by injecting 0.1 ml of an M/1,000 solution. When 0.1 ml of an M/100 solution was injected, its effect was intense.

Smaller vasconstrictor effects were obtained as a rule when asym-dimethylguanidine was given in corresponding amount. Guanidine itself was less active still

Effect of 'Priscol — In ten experiments benzyliminazoline ('Priscol') hydrochloride was perfused as a 1 5,000 solution in Ringer-Locke

after control injections of methyl isothiourea and of adrenaline had been given. Within a few minutes the response to even a large (0 1 μ g) dose of adrenaline was "reversed" Nevertheless, vasoconstriction was still brought about when methyl isothiourea was injected (Fig. 1d). Its action did not seem to be affected appreciably by the priscol nor did that of guanidine or of asym dimethyl guanidine

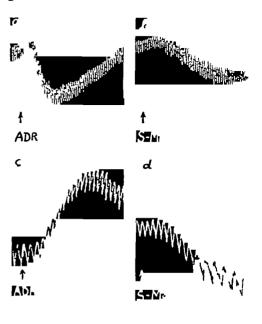


FIG 1—Perfused rabbit ear preparation Upper tracings show the normal effect of (a) adrenaline (0 01 μg at ADR) and (b) methyl isothiourea (0 1 ml of M/5,000 at S-Me) Lower tracings show the effect of (c) adrenaline (0 1 μg at ADR) and (d) methyl isothiourea (0 1 ml of M/5,000 at S-Me) after a 1 5,000 solution of priscol has been perfused for about ten minutes

Effect of "Anthisan -Unlike priscol, the antihistamine compound N-p-methoxybenzyl-Ndimethylaminoethyl - a - aminopyridine ("Anthi san') maleate antagonized the vasoconstrictor response to methyl isothiourea in concentrations which left the blood vessels still fairly responsive to adrenaline In all of seven experiments it soon diminished the response to a test dose of methyl isothiourea when perfused in M/1,000 solution (Fig 2) Ultimately even so large a dose as 01 ml of M/10 methyl isothiourea failed to Slight vasodilatation produce vasoconstriction was obtained occasionally with the isothiourea, but there was nothing comparable to the "reversal" seen with adrenaline after priscol

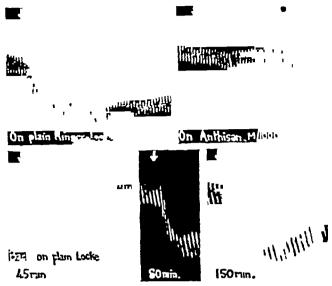


Fig 2—Perfused rabbit ear preparation Methyl isothiourea sulphate (0 1 ml of M/100 given at the arrows) Its effect is practically suppressed when anthisan (M/1,000) is added to the perfusing Ringer-Locke solution. Even a very large dose of methyl isothiourea (0 1 ml of M/10 at the dot) produces hardly any vasoconstriction.

Anthisan in this amount was found to antagonize the vasoconstrictor action of adrenaline too, although not so completely, vasoconstriction could still be produced in response to a large $(2 \mu g)$ dose of adrenaline at a time when the effect of even a 1 mg dose of methyl isothiourea was quite suppressed. Sensitivity to methyl isothiourea was very slowly restored when ordinary Ringer-Locke solution was perfused again in place of the anthisan solution.

Isolated gut

Slight but definite antagonism between anthisan and methyl isothiourea was also noticed in organ

bath experiments with guinea-pig ileum. Methyl isothiourea tends to increase the tonus of the strip when it is added to make a bath concentration of M/10,000 or higher. This effect was found to be suppressed more or less completely when anthisan was given before the isothiourea in concentrations of M/10,000 and upwards.

The tonus-increasing action of methyl isothiourea on isolated rabbit ileum was not much impaired by a concentration of atropine (M/10,000) sufficient to render the muscle insensitive to even large amounts of acetylcholine. When guinea-pig ileum was used in place of rabb.t ileum, however, it was found that this same concentration of atropine greatly reduced the response to a test dose of methyl isothiourea.

In the presence of methyl isothiourea the response of guinea-pig ileum to a test dose of histamine may be increased or decreased, according to the conditions employed A decrease was always obtained when the dose of isothiourea was of itself sufficient to produce a considerable rise Higher homologues like n-butyl and n-heptyl isothiourea were found to exert a much stronger spasmolytic action, dose for dose slightly increased response to a small dose of histamine was seen occasionally with slightly smaller concentrations of methyl isothiourea (of the order of M/10,000) Potentiation of the response to histamine by methyl isothiourea was observed quite regularly when the strip had first been rendered moderately insensitive to histamine by treatment with anthisan In these circumstances, doses of methyl isothiourea or of asymdimethylguanidine which had little effect themselves on muscle tone increased appreciably the response to a test dose of histamine

TABLE I EFFECTS OF ISOTHIOUREAS UPON THE BRONCHOCONSTRICTOR RESPONSE TO HISTAMINE

Substance	Dose	No of exps in which histamine response was				
	Dose	Increased	Unchanged	Decreased		
Methyl isothiourea	0 1 ml M/10 0 25 ml ,,	5 4	4 4			
n-Hexyl ,,	0 1 ml ,, 0 25 ml ,,			3		
n-Heptyl ,,	0 1 ml ,, 0 25 ml ,,		1	2 3		
Tetramethylene di-150thiourea	0 1 ml M/20 0 25 ml ,,	1	2 1	2		

Guinea-pig lung preparation

Slight transitory bronchoconstrictor responses were produced in the following experiments by injecting small test doses of histamine acid phosphate (2-10 μ g/kg) at regular intervals of five minutes or more. The response to histamine was altered by various isothioureas as indicated in Table I. In about half the experiments performed with methyl isothiourea, the bronchoconstrictor effect of histamine was definitely potentiated (Fig. 3), it was never reduced. However, higher

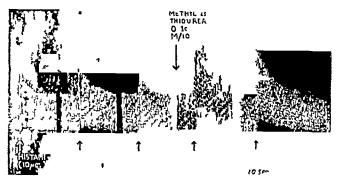


Fig. 3—Guinea-pig lung preparation. The bronchoconstrictor effects were produced by 10 μg doses of histamine acid phosphate injected at 10 min intervals. The response to histamine is increased after a dose of methyl isothiourea (0.1 ml. of an M/10 aqueous solution of the sulphate).

homologues usually produced anti-histamine effects when given in the same range of doses Sometimes the effect of histamine was reduced by as much as 60-80 per cent

Methyl isothiourea had no distinctive effect per se, exceptionally it caused slight bronchoconstriction. In two of the experiments with n-heptyl isothiourea, there was considerable bronchoconstriction after its injection. Unfortunately the interpretation of some of these results was made difficult because spontaneous respiratory movements were resumed soon after the isothiourea was injected. In most instances the compounds had no direct effect on the bronchioles.

Isolated rat uterus

It was confirmed that histamine has an inhibitory action on non-pregnant rat uterus. It should be added, however that doses of the order of 1-5 ug/ml were required to demonstrate any effect, although the horns were quite sensitive to adrenaline and acetylcholine.

Methyl isothiourea was tested several times on each of eight preparations in concentrations of from M/10,000 to M/2 000 Usually it had no

effect at all, sometimes there appeared to be slight stimulation. More definite evidence of stimulation was obtained with n-butyl isothiourea. In four of six experiments a bath concentration of M/2,000 caused a slight increase in the tone and motility of the strip

Liffects on striped muscle preparations
Isolated frog rectus abdominis muscle

In ten successive experiments the addition of methyl isothiourea (M/500) to the test dose of acetylcholine resulted in an enhanced response of the muscle. The isothiourea alone did not increase muscle tone in four control experiments, but its potentiating activity became manifest in these too when the Ringer solution containing methyl isothiourea sulphate was replaced by the test solution of acetylcholine.

Potentiation of the response to acetylcholine was also observed regularly in experiments with ethyl isothiourea and with asym-dimethylguanidine. On the other hand, the long-chain amidine derivative n-hexyl isothiourea was found to antagonize the action of acetylcholine even in a dilution of M/5,000

Rat phrenic nerve-diaphragm preparation

An enhanced response to a short-acting (0.5 msec) electrical stimulus—a slightly supermaximal shock given indirectly every 10 seconds—was obtained regularly after the injection of an M/10 solution of one of the following salts into the 100 ml bath guanidine hydrochloride (0.5-2.0 ml), methylguanidine sulphate (0.5-1.0 ml), methyl isothiourea hydrochloride and sulphate (0.25-1.0 ml), and ethyl isothiourea hydrobromide (0.25-0.5 ml). The phenomenon was seen almost immediately after the addition of methyl or ethyl isothiourea, but not for some minutes after the addition of guanidine or of asym-dimethylguanidine.

Potentiation after methyl isothiourea was still observed in experiments in which a longer-acting electrical stimulus (35 msec—the maximum for the instrument) was applied or in which the rate of stimulation was ten times faster

The higher homologues n-butyl and n-hexyl isothiourea reduced the amplitude of the twitch when added in the same range of doses as methyl isothiourea. Transitional behaviour was displayed by n-propyl isothiourea whereas moderate concentrations (M/2,000-M/500) somewhat increased the amplitude of the twitch, higher concentrations decreased it

Interaction with d-tubocurarine -A dose of 1 μg/ml of d-tubocurarine chloride was found sufficient to curarize the preparation, the muscle could now be made to contract only by stimulating it directly Given at this point guanidine (M/500) increased considerably the contractions brought about by direct stimulation, but indirect stimulation indicated that this potentiating effect of guanidine was due in part to an anti-curare action, such an action was demonstrated directly in six experiments Methyl isothiourea was found to behave similarly when given in a concentration of M/4,000 Its potentiating action was seen best when the dose of d-tubocurarine administered was one which produced only partial block

When the dose of d-tubocurarine was increased to 2 µg/ml, no anti-curare action could be demonstrated with the above doses of methyl Nevertheless, both isothiourea and guanidine amidine derivatives tended to augment the effect of direct electrical stimuli if given under these

D.→I. (c.) (a) Mijiii

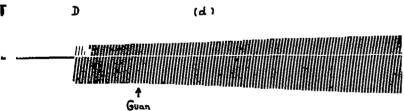


Fig. 4—Rat diaphragm preparations—I indicates indirect and D direct stimulation (a) The effect of maximal indirect shocks (17 m amp, 05 msec shocks at 10 sec intervals) is abolished by a 200 μ g dose of d-tubocurarine (b) Same preparation Contractions now elicited by direct electrical stimulation Methyl isothiourea hydrochloride (M/4,000 at the arrow) enhances the response to these, although (c) indirect stimulation shows that the preparation remains fully curarized After two washings, sensitivity to indirect stimuli slowly returns (d, e) A similar experiment with guanidine hydrochloride (M/500 at the arrow) There is a 10 min interval between strips (d) and (e)

conditions, methyl isothiourea to nearly its usual extent but guanidine sometimes hardly at all (Fig 4)

Influence of calcium ion concentration — A reduction in the effective concentration of calcium ions in the bath was brought about (1) by adding sodium oxalate solution to the bath, (11) by adding sodium citrate solution, or (iii) by replacing the

ordinary Ringer-Tyrode solution in the bath with a solution containing less than the normal amount of calcium chloride It was noted, confirming McDowall (1949), that a moderate reduction in the calcium ion concentration of the bath usually increases the muscle twitch, whereas a large reduction invariably decreases it In the latter instance, adding calcium chloride solution restored the amplitude of the twitch, the normal effect of an increase in calcium ion concentration was to reduce the twitch

In six experiments guanidine (M/500) was added to Ringer-Tyrode solution containing half the normal amount of calcium The average increase in the size of the maximal twitches in these six experiments was definitely less than that observed in seven control experiments in which the same dose of guanidine was added to normal Ringer-Tyrode solution Raising the calcium content of the bath was less effective for counteracting the action of guanidine One of the most striking

> features of this set of experiments was the persistence of the guanidine effect Even ten or fifteen washings failed to restore the normal reaction of the preparation Consequently little could be learned from the effect of a second dose

Sımılar experiments were carried out with methyl isothiourea As a test dose, 05 ml of an aqueous M/10 solution was injected into the 100 ml bath In ordinary Tyrode solution this dose of methyl thiourea caused a slow steady increase in the size of the twitch in almost all of some twenty trials The response to the isothiourea was reduced — sometimes

suppressed—when the calcium ion concentration was lowered sufficiently to reduce the amplitude of the twitch, but it was not much affected by doubling or quadrupling the calcium content of the bath

Interaction with potassium—Raising the potassium ion concentration of the Ringer-Tyrode solution in the bath at first potentiates increasingly the response to a maximal indirect shock, then reduces it—just as does a typical amidine derivative when it is given in increasing amount

It was observed that after two 20 mg doses of potassium chloride (at which time a further increase in the potassium ion concentration of the 100 ml bath could no longer increase the size of the twitch), test doses of methyl or ethyl isothiourea were unable to cause potentiation Conversely, a test dose of potassium chloride given after a large dose of a short-chain amidine derivative produced much the same sort of effect as would be expected if excess potassium were already present In experiments with guanidine this change in the reaction of the preparation was still seen after eight or ten washings-which is in accord with the persistence of other effects of guanidine Amongst alkyl isothioureas, the n-hexyl derivative was found to antagonize the potentiating effect of potassium chloride more effectively than those with shorter side-chains

Cat sciatic nerve-gastrocnemius preparation

Methyl *iso*thiourea sulphate was tested in doses of 2-5 mg/kg on eleven cats. Almost as soon as the drug solution entered the iliac artery the 'maximal' contractions produced by stimulating

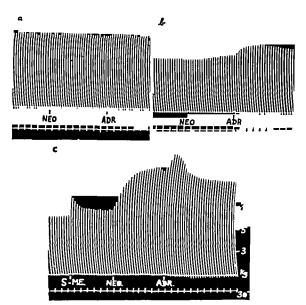


FIG 5—Cat \$\to\$ 2.8 kg, under chloralose gastrocnemius muscle preparation Contractions elicited by maximal shocks at 10 sec intervals. Neostigmine given at 90 min intervals (doses of 10 \(mu\)g at AEO; followed 2-5 min later by adrenaline (10 \(mu\)g at ADR) Note the potentiated response to neostigmine after the administration of methyl isothiourea sulphate 10 mg at S-Me)

the muscle through the sciatic nerve six or twelve times a minute were increased, usually by some 10-20 per cent (Fig 5c) When given in the same range of doses guanidine hydrochloride had practically no effect, while *n*-hexyl *iso*thiourea hydrobromide decreased the height of the contractions

If muscle contractions were first decreased some 20-30 per cent by giving a small dose of d-tubocurarine, methyl isothiourea caused more striking potentiation than usual. If, however, enough d-tubocurarine was given to produce an 80-90 per cent decrease, the effect of a subsequent dose of methyl isothiourea was relatively slight

In eight experiments small doses of neostigmine (3-6 µg/kg) were given every 90 minutes. Even this long interval between doses was usually insufficient to prevent cumulation entirely. However, cumulation alone seemed inadequate to explain the greatly enhanced effect of a test dose of neostigmine given after either methyl isothiourea or guanidine (Fig 5). In three of the four experiments made with each compound it appeared certain that the amidine derivative had po entiated the effect of the neostigmine.

Adrenaline had little or no effect on this preparation when given in a dose of 3-5 mg/kg, but after the administration of neostigmine the test dose of adrenaline usually produced a considerable increase in the size of the muscle twitches. No corresponding effect was seen in parallel experiments with guanidine or methyl isothiourea. However, in several preparations in which the adrenaline caused only a decrease in the size of the contractions, this depressant effect was seen to be enhanced after methyl isothiourea had been given

DISCUSSION

In their effects upon smooth muscle preparations amidine derivatives like methyl isothiourea and guanidine resemble histamine rather than This is shown especially by the adrenaline experiments with blocking agents. Whereas the vasconstrictor action of methyl isothiourea on the rabbits ear preparation was antagonized appreciably by "Anthisan (an anti-histamine drug), it was hardly affected by "Priscol" (which is pre-Not all actions dominantly an anti-adrenaline) of histamine on muscle are reproduced, how-The amidine derivatives tested showed no tendency to relax rat uterus They differed from histamine moreover in acting on striped muscle almost as strongly as on smooth

When effects on striped muscle preparations are taken into consideration it becomes evident that

these amidine derivatives resemble potassium more closely than histamine like potassium, they potentiate the effect of "maximal" electrical shocks delivered to rat diaphragm muscle through the phrenic nerve, they also antagonize the effect of low concentrations of d-tubocurarine on the rat diaphragm preparation whilst having their own effects reduced by higher concentrations d-tubocurarine—which suggests that they compete with it for the same receptors (cf Quilliam and Moreover, potassium salts in Taylor. 1947) moderate excess resemble typical amidine derivatives in causing the contraction of most types of smooth muscle, they also have a vasoconstrictor action which is not affected appreciably by the treatment with ergotoxine, apart from one caused by the liberation of adrenaline (Mathison, 1911, Knoefel and Alles, 1938, Fastier and Smirk, 1947 Sugawara and Tada, 1927)

It must be added that the majority of these effects can also be reproduced by a reduction in calcium ion concentration. Thus in comparing the effects of guanidine on frog rectus muscle with those of calcium deprivation, Harvey (1940) observed that "both bring about a sensitization to potassium ions, their action is antagonized by excess of calcium, and both lead to a 'spontaneous' activity" The close correspondence between symptoms of guanidine poisoning and those of parathyroid tetany has long been known (Paton and Findlay, 1917)

Nevertheless, so closely linked are the physiological actions of potassium and calcium that Harvey and others have been content to attribute the pharmacological effects of guanidine to changes in ionic balance rather than to anything so specific as calcium lack or potassium excess Neither possibility is ruled out by the above results, but it seems significant that the chemical relatives of methyl isothiourea which have been found to resemble it at all closely in pharmacological properties are those whose salts ionize freely to yield small cations (Fastier, 1948) order to explain the effects of these various bases in terms of "potassium excess" one need only assume that they can simulate more or less closely the action of potassium ions, unlike their higher homologues, which show a far greater tendency to produce only inhibitory effects (Fastier and Reid 1948) It is far less easy to explain how a reduction in the effective concentration of calcium ions might be brought about

Whatever be the precise explanation, it is obvious that amidine derivatives have a deep-seated action on muscle. A diversity of muscle-

contracting agents have their effects modified in the presence of amidine derivatives. Anti-histamine effects on gut and bronchioles have been observed with large doses of amidine derivatives and potentiation of histamine effects with smaller doses. Analogous effects upon sensitivity to the vasoconstrictor action of adrenaline have been described by Fastier and Reid (1948). Pharmacological effects of acetylcholine too may be modified by typical amidine derivatives.

It has been known for some considerable time that guanidine can potentiate the action of acetylcholine on striped muscle (Frank, Stern, and Nothmann, 1921, Harvey, 1940) The finding that bases like asym-dimethylguanidine, methyl and ethyl isothiourea also possess this property is hardly surprising considering the numerous other analogies that have been noted in their pharmacological behaviour (Fastier and Smirk, 1947) That guanidine can sensitize frog rectus muscle to potassum as well as to acetylcholine has been shown by Harvey (1940) The ability of guanidine to potentiate the effects of a variety of musclecontracting agents is further evident from results of Camis (1909), Paton and Findlay (1917), Fühner (1920), and Kato (1939) on striped muscle, and of Fühner (1917), Burns and Watson (1920), Godeaux (1942), and García and Perdomo (1946) on smooth muscle preparations In the rat phrenic nerve-diaphragm preparation the increased response to "maximal" electrical shocks applied indirectly may well depend solely upon an enhanced sensitivity to the acetylcholine liberated at myoneural junctions However, this leaves unexplained the observation that some potentiation can still be obtained after curarization

Guanidine has been found to be of some value for the treatment of myasthenia gravis (Minot, Dodd, and Riven, 1939), though why it affords relief to myasthenics is far from evident Guanidine does not inhibit cholinesterase (Minot, 1939). Thompson and Tice, 1941) Therefore it might be concluded that the action of this drug is quite different from that of neostigmine, but Thompson and Tice question such a view They point out inter alia that the duration of relief of symptoms in myasthenia gravis after neostigmine follows the change in serum potassium more closely than it does the decrease in activity of cholinesterase Like Cumings (1939), they believe that some abnormality of potassium metabolism is present in myasthenia gravis. The above results suggest that potentiation of the effect of acetylcholine is but one manifestation of a more general action of guanidine on striped muscle, and possibly it is

not this but some related effect which is of therapeutic value in myasthenia gravis. It may be remarked in conclusion that, as several other amidine derivatives have been shown to resemble guanidine closely in their effects upon striped muscle preparations, some of them might also prove of clinical value

SUMMARY

- l Effects of methyl isothiourea and guanidine salts have been studied on perfused rabbit cars, isolated strips of rabbit and guinea-pig ileum, isolated rat uterus, guinea-pig bronchioles (Konzett-Rossler technique), isolated frog rectus muscle, cat sciatic nerve-gastrocnemius, and rat phrenic nerve-diaphragm preparations. A few experiments have also been performed with salts of other alkyl isothioureas and of methyl- and asym-dimethyl-guanidine.
- 2 The short-chain amidine derivatives tend to increase the tonus of smooth muscle, though their effect on some preparations is negligible. The vasconstrictor response to methyl isothiourea, unlike that of adrenaline, is not antagonized appreciably by "Priscol," but both the vasoconstrictor action of methyl isothiourea and its tonusincreasing action on gut are partially antagonized by the anti-histamine drug "Anthisan"
- 3 The effects of methyl isothiourea and of guanidine on striped muscle preparations resemble those of potassium in excess. They are able to potentiate the effect of maximal electrical shocks applied indirectly to the rat diaphragm preparation, they exert an appreciable anti-curare action, and their potentiating action on the rat diaphragm preparation is not readily antagonized by d-tubocurarine
- 4 Several of the short-chain am dine derivatives have been shown to enhance the response of frog rectus muscle to test doses of acetylcho ine Potentiation of effects of histamine on gut and bronchioles has also been observed under conditions described in the text References to yet other potentiating actions of amidine derivatives on muscle are given
- 5 The mode of action of these compounds is discussed, particularly in relation to that of histamine and of potassium

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SOME RELATIONSHIPS BETWEEN ANTI-NICOTINE ACTIVITY AND SPECIFIC ANTAGONISMS

BY

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Besides atropine, new synthetic compounds with parasympathicolytic and spasmo'ytic activity have been shown recently to possess therapeutic value in Parkinson's disease, these are diethylam.noe.hyl-1 - phenyl - cyclopentane - 1 - carboxylate (DPCPC) (Grunthal, 1946, Hartmann, 1946, Schwab and Leigh, 1949) and according to Sigwald et al (1946) and Bovet et al (1947, 1948) N-diethylaminoethylphenothiazine (DPT) The first is parasympathicolytic and papaverine-like (Domenjoz, 1946, Frommel et al, 1949) and exhibits at the same time some antihistamine (Frommel et al., 1949), peripheral curare-like (Domenjoz, 1946), and local anaesthetic (Fleisch and Baud, 1948) properties In addition to these effects, Heymans et al (1948a and b, 1949b and c) have demonstrated that DPCPC is a powerful anti-nicotine agent because it suppresses all the toxic effects of high doses of this alkaloid The same authors have recently shown that DPT also exerts a strong anti-nicotine activity in protecting dogs against 100-200 lethal doses of nicotine (1949a, b, and c)

The very complex pharmacological activity of nicotine is generally divided into peripheral effects on muscular fibres and neuromuscular junctions, effects on autonomic ganglia, and effects on the central nervous system Further and at each level, the action of nicotine depends on the alkaloid concentration and on the degree of intoxication, so that these can also be classified into motor and inhibitory effects (Bovet et al, 1948, Goodman and Gilman, 1946) Corresponding to these modes and sites of action, nicotine can thus produce some effects on synaptic activity which, according to Langley and Dickinson (1889), can be used as a test of nicotine-like or anti-nicotine activity. On the other hand many other effects such as those on the respiratory, cardiovascular or central nervous system, or on isolated organs—e.g., intestine, vessels, or heart—do not show a clear relationship to the usual physiological stimulations As it was supposed that the therapeutic activity of some compounds in Parkinson's disease might be related to their ganglionic-blocking effect on the tonic centres of the extrapyramidal tracts or on the cholinergic transmission (Sigwald et al, 1946, Bovet et al, 1947, 1948), we thought that it would be interesting to investigate more closely the relationship between anti-nicotine effect and parasympathicolytic or autonomic activity For a better discrimination of such antagonisms, it seemed also necessary to extend our investigations to compounds of diverse chemical constitution possessing some specific activity such as antagonism of parasympathetic or sympathetic effects, spasmolysis, ganglionic-blocking, local anaesthesia, and even central depression

We tested at first the overall antagonism against nicotine on the whole animal by determining protective indices

METHOD

Mice weighing about 20 g received intravenously a toxic dose of nicotine (0 00125 g/kg) which produced convuls ons, paralysis, and death in all animals. One hour previously, doses of the presumed protective agent were given subcutaneously to groups of 6-10 mice, and the ED50, protecting 50 per cent of animals, was determined according to Miller and Tainter's method (1944) which allows if necessary a statistical treatment of the results

In a first series of experiments we tested the antinicotine effect of atropine as a true parasympathicolytic agent compared with DPCPC and DPT and of two synthetic compounds with neurogenic and myogenic activity (Meier, 1936, Johnson and Reynolds 1937, Meier and Hoffmann, 1940, Graham and Lazarus 1940 Tripod, 1949)—viz, "Trasentin (diethylaminoethylester of diphenylacetic acid) and

Trasentin-H" (diethylaminoethylester of phenylcyclohexylacetic acid) A specific sympathicolytic agent like ergotamine and tetraethylammonium

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bromide (TEA), which shows some anti-nicotine effect (Boelaert, 1948) besides its marked ganglionic-blocking action (Acheson et al. 1945, 1946a and b, Trendelenburg, 1923), were also investigated Procaine and cocaine were tested because the former possesses, if not a pure ganglion-blocking effect (Bovet et al. 1948), at least some parasympathicolytic and anti-nicotine properties (Hazard et al. 1942a and b, Moore et al. 1948, Haimovici, 1948, Soehring and Hessler 1949). Finally d tubocurarine as a nerve muscle blocking agent (Feldberg and Lin, 1949) and phenobarbitone as a general depressant of the central nervous system were used

In a second series of determinations we tested in the same manner the anti-acetylcholine effect of all these compounds against an intravenous dose of acetylcholine (0 020 g /kg) which produces convulsions paralysis, and death in all animals

RESULTS

Table I gives the ED50 values for the antinicotine and anti-acetylcholine activities of all these agents

TABLE I

ANTI-NICOTINE AND ANTI-ACETYLCHOLINE ACTIVITY OF VARIOUS AGENTS ON MICE

Class of drug	Drug	Anti- nicotine ED50 g /kg	Anti- acetylcholine ED50 g /kg
Parasympathi- colytic and spasmolytic	Atropine DPT DPCPC Trasentin Trasentin-H	>0 800 0 014 0 031 0 031 0 085	0 009 0 130 0 013 0 035 0 018
Sympathicolytic	Ergotamine	>0 100	>0 100
Ganglion-block- ing	Tetraethyl- ammonium	0 028	>0 200
Local anaes- thetic	Cocaine Procaine	0 017 0 100	>0 200 >0 400
Peripheral- blocking	d-Tubocura-	>0 00075	>0 00075
Central	Phenobarbi- tone	0 007	>0 100

These results show clearly that the general toxicity of nicotine can be antagonized by spasmolytic and ganglion-blocking agents or even by cocaine as well as by a central depressant. These findings seem thus to be related not only to a specific effect but also to various sites of action of the alkaloid. In addition, the lack of activity

of atropine is very striking so that a parasympathomimetic effect of nicotine is unlikely Further, this test also clearly demonstrates that a good anti-nicotine activity is not exclusively exhibited by DPT and DPCPC, since trasentin and to some extent trasentin-H can also antagonize the effects of nicotine

On the other hand, an anti-acetylcholine activity is shown only by atropine and by spasmolytic agents which are known to possess parasympathicolytic properties like DPT (Bovet et al, 1947, 1948, Gordon, 1948, Heymans et al, 1949a, b, and c), DPCPC (Domenjoz, 1946, Bovet et al, 1948, Heymans et al, 1948a and b, Frommel et al, 1949), trasentin, and trasentin-H (Meier, 1936, Meier and Hoffmann, 1940, Graham and Lazarus, 1940, Bovet et al, 1948, Tripod, 1949)

TABLE II

RELATIONSHIP BETWEEN ANTI-NICOTINE AND ANTIACETYLCHOLINE ACTIVITIES

Drug	Index anti-acetylcholine potency
Atropine	<0.01
DPT	9 30
DPCPC	0 42
Trasentin	1 13
Trasentin-H	0 21

The ratios between the ED50 values for the antinicotine and anti-acetylcholine activities (Table II) demonstrate that these properties are clearly independent in these spasmolytic compounds

Since an anti-nicotine activity on the whole animal is exhibited by various compounds, which are neither chemically nor pharmacologically related, it seemed necessary to compare the effect of nicotine with typical autonomic effects in a simpler test

Acetylcholine has a consistently stimulant effect on isolated rabbit and guinea-pig ileum. Nico tine on the contrary produces on the intestine a contraction as described by Trendelenburg (1917), Alvarez (1918a and b), von Oettingen et al (1928), and recently by Feldberg and Lin (1949), or an inhibition of the activity with higher concentrations according to Langley and Magnus (1905). This effect can be mixed or biphasic (Alvarez 1918), and depends on the dose, the anatomical localization of the intestinal strip, the animal species, as well as on the experimental technique (Alvarez, 1918, Raymond-Hamet 1930 Bovet

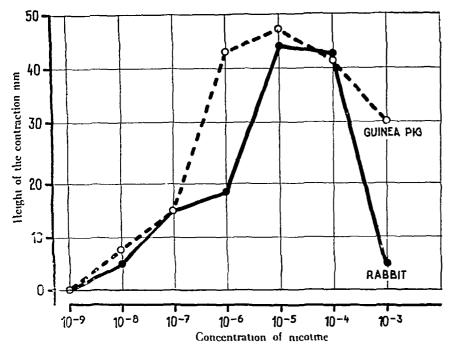


Fig 1 —Relationship between concentration of nicotine and height of contraction of the isolated guinea-pig and rabbit ileum

et al, 1948) It seemed therefore necessary to establish a test in which a typical motor effect of nicotine would be produced

METHOD

Isolated strips of rabbit or guinea-pig ileum were kept at 0° during four hours and afterwards suspended in a 50 cc bath containing oxygenated Tyrode. The bath temperature was 38° C and a frontal writing lever was used. The concentrations mentioned in the text are the final concentrations in the bath.

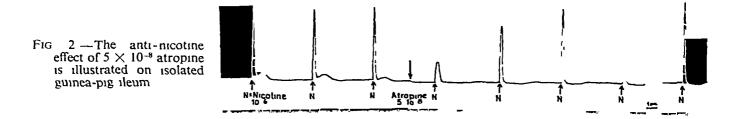
RESULTS

The mean response-dose curves for nicotine on the rabbit and guinea-pig ileum are depicted in Fig 1

Guinea-pig ileum seemed suitable for the testing of a nicotine antagonism because its motor effect has a wider range, moreover, a biphasic response is only elicited by concentrations higher than 10-4, and the contractions can be reproduced quite regularly without tachyphylaxis, at least with low concentrations as already described by Feldberg and Lin (1949) Rabbit ileum, on the other hand, reacts more irregularly to the alkaloid and shows even in low concentrations, for example 10-7, a biphasic or polyphasic effect

The antagonistic activities of various agents were tested on

isolated guinea-pig ileum against a final nicotine concentration of 10 ° The anti-nicotine effects recorded after the prophylactic treatment with the antagonist could then be measured and the inhibition statistically treated for "all or none" (Morrell et al, 1940, Miller et al, 1948) or graded responses (Schild, 1942, Holton, 1948, Tripod, 1949) Figs 2 and 3 illustrate the antagonistic effects of atropine and trasentin-H The values of the concentrations producing a 50 per cent anti-nicotine effect (ED50)



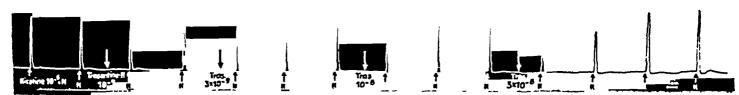


Fig 3—Trasentin-H produces even in a concentration of 10 ° a small anti-nicotine effect on isolated guinea-pig illum. The inhibition of the motor effect of nicotine is marked with 3 × 10 °.

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TABLE III
ANTI-NICOTINE ACTIVITY OF VARIOUS AGENTS ON ISOLATED
GUINEA-PIG ILEUM

Class of drug	Drug	Anti- nicotine ED50	Relative
Parasympathi- colytic and spasmolytic	Atropine DPT DPCPC Trasentin Trasentin-H	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	100 20 100 33 100
Sympathicolytic	Ergotamine Priscol	10-5 10 5	02
Ganglion-block- ing	Tetraethyl- ammonium	>10 4	<0 02
Local anaes- thetic	Cocaine Procaine	>10 ⁴ 10- ⁵	<0 02 0 2
Peripheral- blocking	d-Tubocurarine	3 × 10-4	0 006
Central	Barbitone Phenobarbitone	>10 ⁻⁴ 10 ⁻⁴	<0 02 0 02

have been graphically evaluated from several experiments and are listed in Table III, where the relative potency of atropine is put as 100

The greatest anti-nicotine activity is strikingly shown by atropine and the spasmolytic compounds, whereas sympathicolytic, ganglion-blocking, local anaesthetic, and peripheral-blocking

TABLE IV

ANTI-ACETYLCHOLINE ACTIVITY OF VARIOUS AGENTS ON

ISOLATED RABBIT ILEUM

Class of drug	Drug	Anti- acetyl- choline ED50	Relative potency	
Parasympathi- colytic and spasmolytic	Atropine DPT DPCPC Trasentin Trasentin-H	2 3 × 10 ⁸ 4 × 10 ⁻⁷ 2 × 10 ⁷ 4 4 × 10 ⁷ 1 6 × 10 ⁷	100 57 11 5 5 2 14 4	
Ganglion-block- ing	Tetraethyl- ammonium	3 2 × 10-4	0 007	
Local anaes- thetic	Cocaine Procaine	2 3 × 10 ⁴ 2 1 × 10 ⁵	0 01 0 11	
Peripheral- blocking	d-Tubocurarine	>10 *	<0 02	
Central	Phenobarbitone	>10 4	<0.02	

agents as well as central depressants all show an antagonism, but in such high concentrations that their specificity is rather doubtful. In their recent paper Feldberg and Lin (1949) drew attention to the anti-nicotine effect of local anaesthetics and of d-tubocurarine on the guinea-pig and the rabbit ileum, in our experiments on guinea-pig gut, however, this inhibition was found to be much smaller than that of atropine

In the same manner we tested on the rabbit ileum the antagonism of some agents against a final concentration of acetylcholine of 5×10^{-7} . The ED50 values are compiled in Table IV, where the relative potency of atropine is again 100

As expected, only atropine and atropine-like agents are here effective, but the spasmolytic compounds possess different potencies for anti-nicotine and anti-acetylcholine activity as shown by Tables III and IV, although both are produced by rather similar concentrations

Further, we compared various antagonistic effects in order to establish the relationships

TABLE V
RELATIONSHIP BETWEEN ANTI-NICOTINE EFFECT AND OTHER
ANTAGONISMS ON ISOLATED ORGANS

	Indices of specific potencies						
Drug	Anti-nicotine Anti- acetylcholine	Sympathi-	Anti-nicotino Musculo- tropic				
Atropine DPT DPCPC I rasentin Trasentin-H	1 1 4 10 7 3 8	3,000 30 1,000 333 1,000	50,000 100 500 167 150				

between the anti-nicotine specificity, the sympathicolytic activity against 3×10^{-6} adrenaline on the seminal vesicle of the guinea-pig (Brügger, 1945), and the antagonism against 2×10^{-4} BaCl₂ on the rabbit ileum

Calculated ratios of anti-nicotine activities to other antagonistic activities are reproduced in Table V. They show that the anti-nicot ne activity is more closely related to the anti-acetylcholine activity than to any of the other antagonisms

On the isolated guinea-pig ileum it can thus easily be demonstrated that atropine and spasmolytic compounds like DPT, DPCPC, trasentin, and trasentin-H exert a specific antagonism against the nicotinic stimulation, antagonism which is not related to anti-adrenaline or musculotropic properties. A closer relationship is only exhibited with

an anti-acetylcholine effect, so that this point seemed worthy of further investigation. Therefore, we tested the antagonistic effect of these agents against other substances with muscarine-like properties such as arecoline and pilocarpine (Bovet et al., 1948) and determined the various antagonistic ED50 values on the guinea-pig ileum against acetylcholine (10-7), arecoline (3 × 10 8), and pilocarpine (5 × 10 7) (Tables VI and VII)

TABLE VI
ANTAGONISMS ON ISOLATED GUINEA-PIG ILEUM

Drug	Antı-	Anti-	Anti-	Anti-
	acetyl-	nico-	areco-	pilo-
	choline	tine	line	carpine
	ED50	ED50	ED50	ED50
Atropine DPT DPCPC Trasentin Trasentin-H	$\begin{array}{c} 7 \times 10^{-9} \\ 2 \times 10^{-7} \\ 8 \times 10^{-8} \\ 4 \times 10^{-7} \\ 2 \times 10^{-8} \end{array}$	$\begin{array}{c} 2 \times 10^{-8} \\ 10^{-7} \\ 2 \times 10^{-8} \\ 6 \times 10^{-8} \\ 2 \times 10^{-8} \end{array}$	$\begin{array}{c c} 7 \times 10^{-10} \\ 2 \times 10^{-8} \\ 2 \times 10^{-8} \\ 3 \times 10^{-8} \\ 2 \times 10^{-9} \end{array}$	$\begin{array}{c} 4 \times 10^{-8} \\ 3 \times 10^{-8} \\ 6 \times 10^{-8} \end{array}$

TABLE VII
RELATIONSHIP BETWEEN ANTI-NICOTINE EFFECT AND OTHER
ANTAGONISMS ON ISOLATED GUINEA-PIG ILEUM

	Indices of specific potencies					
Drug	Anti-nicotine Anti- acetylcholine	Antı-	Anti-nicotine Anti- pilocarpine			
Atropine DPT DPCPC Trasentin Trasentin-H	0 35 2 0 4 0 6 7 1 0	0 035 0 20 1 00 0 50 0 10	0 1 0 4 1 5 1 0 0 15			

Similar experiments showed that tetraethylammonium, cocaine and procaine, as well as d-tubocurarine, exhibit only an unspecific antagonism against these muscarine-like substances in concentrations higher than 10-3

DISCUSSION

These findings show that anti-acetylcholine, anti-nicotine, anti-arecoline, and anti-pilocarpine properties are closely related but that, nevertheless, differences of specificity occur. In spite of this, it seems that the group of spasmolytics investigated all exhibit the same type of antagonism against a muscarine-like effect.

The site of action of nicotine on the isolated guinea-pig ileum has thus to be considered. Its motor effect could theoretically be due to a

ganglionic excitation by low concentrations of In our experiments, however, it the alkaloid was demonstrated that a specific ganglionic-blocking compound like tetraethylammonium cannot antagonize the stimulation produced by 10 6 nico tine (Table III) As our experiments were done on ice-treated ileum in order to obtain a greater and more regular sensitivity to nicotine, it might be objected that this treatment already "blocks" the autonomic ganglia of the intestine, this is not the case because we obtained the same values as those listed in Table III with the ileum of freshly killed guinea-pigs, with perhaps a little higher sensitivity to cocaine, procaine, and d-tubocurarine which, however, still remains 100-1,600 times smaller than for atropine A usual ganghonic site of action being excluded, the motor effect of nicotine can be explained by special pharmacological properties of the intestinal ganglia of the guinea-pig, or by a peripheral action on the neural elements—e g, a post-ganglionic stimula-The analogy with arecoline and pilocarpine which also have a postganglionic and parasympathomimetic action (Bovet et al. 1948) strengthens this hypothesis Moreover, a post-gangl.onic action of nicotine has already been demonstrated on the blood vessels by Haimovici (1948)

In any case, an anti-nicotine activity on the whole animal seems to be related to various mechanisms involved in the different sites of action of nicotine This effect is not exclusively a property of spasmolytic compounds with therapeutic activity in Parkinson's disease like DPT and DPCPC, since trasentin and trasentin-H equally show a good anti-nicotine effect, and atropine does not show it at all Furthermore, the anti-nicotine activity on isolated organs is comparable to an anti-acetylcholine or anti-muscarine mechanism. and it is again exhibited with high specificity not exclusively by spasmolytic compounds with therapeutic activity in Parkinson's disease thus that the therapeutic activity of DPT and DPCPC is produced by a different and characteristic mechanism, but neither the anti-acetylcholine nor the anti-nicotine mechanism shows a convincing parallelism with therapeutic activity in Parkinson's disease

SUMMARY

I On mice, the toxicity of nicotine can be antagonized by spasmolytic and ganglionic-blocking agents as well as by a central depressant. These anti-nicotine properties seem to be related to various sites of action of the alkaloid and not to a specific blocking of parasympathetic effects.

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On the isolated guinea-pig ileum nicotine produces a muscarine-like stimulation which can be antagonized by atropine and spasmolytic com-These anti-nicotine properties are related more to an anti-acetylcholine or anti-muscarine effect than to a sympathicolytic or musculotropic activity

We are grateful to Mr Brum and Mr Bubendorf for their technical assistance in these experiments

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THE TOLERANCE OF THE DOG TO INTRATHECAL INJECTIONS OF CRYSTALLINE PENICILLIN

BY

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Although it is known that intrathecal administration of penicillin may have an irritant action on the meninges and on the central nervous system (Walker, 1947, Cairns, 1947), no measurements of the degree of tolerance either in the human subject or in the experimental animal are yet available Moreover penicillin is generally injected into the subarachnoid space dissolved in isotonic sodium chloride solution Isotonic sodium chloride solution has itself a well-defined irritant action on the meninges (Bedford, 1946, 1948a, 1948b) therefore essential that this action should be taken into consideration when determining that of peni-In the experiments now reported, a study has been made of the effect of the intracisternal injection of crystalline penicillin in dogs

EXPERIMENTAL PROCEDURE

The general experimental procedure was similar to that described in earlier publications (Bedford, 1946, 1948a), to which reference for details should be made In this series of experiments, the dogs were anaesthetized with ether Crystalline sodium penicillin G(11) ('Avlon," Imperial Chemical Pharmaceuticals, Ltd) was used throughout the experiments. The penicillin was dissolved in sterile distilled water immediately before injection into the cisterna magna, in a few experiments it was dissolved in a 09 per cent (w/v) solution of sodium chloride in distilled water Although solutions of penicillin in distilled water were found to have an average pH of 55, they were in no sense buffered at that pH After the addition of a relatively small volume of cerebrospinal fluid the solutions rapidly assumed the pH of that A constant volume (10 cc) of solution was introduced in all experiments The animals after recovery from the anaesthetic were allowed to survive for six hours, in a few experiments the survival time was 24 hours. Any animal that displayed symptoms of severe irritation of the nervous system after recovery from the anaesthetic was not allowed to Survive

RESULTS

The effect of the intracisternal administration of 100,000 and 20,000 in penicillin

Two dogs weighing 90 kg and 85 kg respectively were each given 100,000 iu penicillin dissolved in 10 cc distilled water and kept under observation while they recovered from the anaesthetic As the effects of the ether wore off, the animals were found to be holding the head rigidly retracted, they repeatedly licked the nose and attempted to scratch the back of the neck with the hind leg There was a profuse flow of Generalized tremors heralded the onset of convulsions which speedily passed into status epilepticus The symptoms displayed by both animals were almost identical Similar effects were obtained after the administration of 20,000 1 u to two dogs weighing 80 kg and 85 kg The animals were destroyed after the onset of general convulsions before complete consciousness had been regained

The effect of the intracisternal administration of 10,000 in penicillin

The experiments were divided into three groups. In the first group the penicillin was injected dissolved in distilled water and the effects on the pressure and cell content of the cerebrospinal fluid determined at the end of 6 hours. The second group of experiments was similar to the first except that the animals were allowed to survive 24 hours after the injection of the penicillin. In the third group, the penicillin was dissolved in 0.9 per cent (w/v) sodium chloride solution before injection. The effect of the introduction of 10,000 in penicillin in distilled water was studied in twelve dogs seven were allowed to survive for 6 hours and five for 24 hours. All animals presented evidence of irritation of the

TABLE I the effect on the cell content and the pressure of the cerebrospinal fluid of 10,000 i u penicillin and of 0.9% NaCl solution

Per	Penicillin in distilled water				Penicillin in distilled water				0 9% NaCl in distilled water		
Wt of dog	White cells per cu mm	Pressur C S		Wt of	White cells per cu mm	Pressure of CSF		Wt of	White cells per cu mm	Pressur C S	
(kg)	6 hr after injection	Before injection	6 hr later	(kg)	24 hr after injection	Before injection	24 hr later	(kg)	6 hr after injection	Before injection	6 hr later
7 5 12 0 9 5 5 0 8 0 9 0 8 5	4,320 4,230 2,400 1,770 6,790 6,880 Destroyed	170 200 130 140 150 110 130	130 160 120 160 160 230	9 0 11 5 7 5 9 5 10 0	340 330 440 170 210	130 140 120 80 100	130 120 90 140 120	9 5 8 5 6 0 10 5 10 0 6 0 10 5	3,250 1,400 3,400 1,070 2,100 2,300 4,500	140 140 80 110 170 125 100	220 170 220 270 160 250 260
Average 8 5	4,398	147	160	9 5	298	114	120	8 7	2,574	123	220

TABLE II

THE EFFECT ON THE CELL CONTENT AND ON THE PRESSURE OF THE CEREBROSPINAL FLUID OF PENICILLIN IN DOSES

LESS THAN 10,000 I U AND OF DISTILLED WATER

Wt of dog	White cells after 6 hr Pressure of CSF		Wt 1 u penicillin of dog in 0 9% NaCl		White cells after 6 hr	Pressure of CSF			
(kg)	water	per cu mm CSF	Before injection	6 hr later	(kg)	solution	per cu mm CSF	Before injection	6 hr later
9 4 8 2 10 0	7,500 7,500 7,500	340 415 600	140 180 130	140 160 160	9 4 12 0 10 5	1,000 1,000 1,000	6,000 7,800 7,500	80 130 120	230 260 200
Average 9 2		450	150	153	8 6	1,000	4,700	120	170
11 0 9 0 10 0	5,000 5,000	170 280 140	180 80 110	180 80 120	Average 10 1	_	6,500	112	215
	5,000	140	110	120		Distilled water			
Average 10 0		197	123	127	10 5		0	160	180
8 0	2,500	120	80	100	11 5		90	150	160
90	2,500	50	80	100	8.5	_	0	120	110
Average 8 5		85	80	100	7 5	_	80	150	170
80	1 000	35	120	140	86	_	0	130	110
9 5 10 0 7 5	1,000 1,000 1,000	40 20 35	170 90 110	170 110 85	Average 9 3		34	142	146
Average 87		32	122	126					

nervous system after recovery from the anaesthetic The symptoms most commonly encountered were weakness of the forelegs in walking, slight head retraction, chattering of the jaws with salivation, and attempts to scratch the back of the head with the hind leg Head shaking was a prominent symptom in some animals With the exception of one dog which passed into status epilepticus after half an hour, convulsions were not observed These symptoms generally disappeared after one hour and the animals appeared normal at the end of the 6-hour and 24-hour recovery periods four experiments the penicillin was dissolved in sodium chloride solution before injection There was evidence of severe irritation of the central nervous system in all four experiments on recovery from the anaesthetic, and the animals were destroyed after the onset of general convulsions within half to one hour of the administration of the penicilin The results of the experiments are summarized in Table I, where, for the purpose of comparison, the effect of the injection of 10 cc of 0.9 per cent (w/v) sodium chloride solution after 6 hours is also indicated The limited number of experiments in each group did not seem to warrant a statistical investigation of the results

The effect of the intracisternal injection of crystalline penicillin in quantities less than 10,000 i u

Penicillin dissolved in distilled water was injected into the cisterna magna in the following doses 7,500 1 u in 3 dogs, 5,000 1 u in 3 dogs, 2,500 1 u in 2 dogs, 1,000 iu in 4 dogs, and 1,000 iu in sodium chloride solution was injected in 4 dogs The duration of the experiments was 6 hours The effect on the cell count and on the pressure of the cerebrospinal fluid is indicated in Table II were symptoms of irritation of the nervous system in all experiments except after the injection of 1,000 ıu after 2,500 1 u these symptoms were barely perceptible Although the injection of 1,000 i u in sodium chloride solution caused the appearance of a considerable number of polymorphonuclear leucocytes and a rise in the pressure of the cerebrospinal fluid, the animals presented no symptoms of irritation of the nervous system It is evident that the white cell content and the pressure of the cerebrospinal fluid give no indication of irritant action on nerve tissue

Discussion

In the above experiments, dogs of an average weight of 8.7 kg were able to tolerate an intracisternal injection of 1000 i.u. penicillin in

10 cc distilled water without displaying symptoms of irritation of the nervous system Although the pressure of the cerebrospinal fluid remained unchanged after 6 hours, polymorphonuclear leucocytes were invariably present, but their number never exceeded fifty per cumm of cerebrospinal fluid (Table II) These findings contrast markedly with the effects which followed the injection of a similar volume of normal saline solution (Table II) Although symptoms of irritation of the nervous system were not observed after the injection of normal saline solution, the pressure of the cerebrospinal fluid was raised and the polymorphonuclear leucocytic content of the cerebrospinal fluid averaged 2,574 cells per cu mm at the end of the experiments

It will be seen from Table II that 1,000 1 u penicillin dissolved in normal saline solution provoked the appearance of an even greater number of leucocytes and also a rise in the pressure of the cerebrospinal fluid The unsuitability of normal saline solution as a solvent for penicillin required for introduction into the subarachnoid space is well illustrated by the experiments in which 10,000 i u penicillin were administered In four experiments in which the penicillin was injected dissolved in normal saline solution, the animals developed general convulsions after half to one hour and were destroyed On the other hand, convulsions occurred in only one animal out of a series of twelve when the penicillin was dissolved in distilled water, although there was evidence of irritation of the nervous system in every instance

The irritant action of penicillin on the nervous system seems to be of relatively short duration Marked evidence of irritation may be present on recovery from the anaesthetic, as was observed after the introduction of 10,000 iu in distilled water, yet with the exception of one animal that had to be destroyed owing to the onset of general convulsions the remainder appeared normal one hour later Similarly the number of polymorphonuclear leucocytes in the cerebrospinal fluid 24 hours after the injection of 10,000 iu penicillin was of the same order as that found after the injection of distilled water and considerably less than after the injection of normal saline (Bedford 1948a) These findings suggest that penicillin may disappear relatively rapidly from the nervous system after intracisternal injection

It is interesting to note that the injection of normal saline causes the appearance of considerably more leucocytes than the injection of crystalline penicillin in a dose capable of exciting symp oms of irritation of the nervous system. As already stated, a rise in the pressure of the cerebrospinal fluid followed the inject on of normal saline which was never found after the injection of pencillin in distilled water. These findings would seem to indicate that the irritant action of sodium chloride solution is restricted mainly to the covering membranes of the nervous system and possibly to the arachnoid villi. Pencillin, on the other hand, rapidly penetrates the membranes, with the production of relatively little irritation. Its main action is on the nerve cells and their processes rather than on the supporting tissue.

Attempts to discover whether degree of dilution or rate of administration have any influence on the effect produced by a given dose of penicillin have so far yielded indeterminate results. Owing to the small size of the cisterna magna of the dog, it would seem probable that better results would be obtained by making observations on the human subject

SUMMARY

A study has been made of the effect of the injection of crystalline penicillin into the cisterna magna of the dog. The duration of the experiments was 6 hours except in a few instances, where it was 24 hours. The injection of 1,000 i u penicillin in distilled water did not produce symptoms of irritation of the nervous system, the number

of polymorphonuclear leucocytes in the cerebrospinal fluid after 6 hours was no greater than after the injection of a similar volume of distilled water, and there was no rise in the pressure of the cerebrospinal fluid No symptoms of irritation of the nervous system followed the injection of a similar dose of penicillin dissolved in 0.9 per cent (w/v) sodium chloride solution, although the cerebrospinal fluid contained a considerable number of polymorphonuclear leucocytes and its pressure was raised Doses of penicillin greater than 10,000 i u invariably caused convulsions, and this also occurred when 10,000 i u were injected dissolved in sodium chloride solution sions, however, occurred in only one of twelve dogs when 10,000 i u were injected dissolved in distilled water, although there was evidence of irritation of the nervous system in every instance A rise in the pressure of the cerebrospinal fluid never followed the injection of penicillin in distilled water

The significance of these findings is discussed

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STUDIES WITH A RADIOACTIVE SPINAL ANAESTHETIC

RY

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The need for information about the fate of a spinal anaesthetic after its introduction into the spinal theca was early recognized (Miller, 1901), but many years elapsed before the problem was seriously studied by a number of workers, including Stout (1929), Kustner and Eissner (1930), Koster et al (1936, 1938, 1939), Bullock and Macdonald (1938), and Shields (1942) In the main these workers agreed that after the intrathecal injection of procaine there was a rapid initial drop in local concentration with a subsequent period of gradual decrement

Evidently an anaesthetic departs from the subarachnoid space, but what is its route of departure? There are various theoretical possibilities

- (1) Cephalic transit in the spinal cerebrospinal fluid to the cranial subarachnoid space with possible absorption by the cranial arachnoid villi
- (2) Absorption by spinal cord and/or nerve roots with or without a period of "fixation" in these tissues
- (3) Transport via the lymphatic drainage of the spinal theca
- (4) Absorption into the venous drainage of the spinal theca either directly or *via* spinal arachnoid villi (Elman, 1923)
 - (5) A combination of any of the foregoing

It is proposed to describe a study of the fate of a spinal anaesthetic with special reference to its ultimate distribution among the tissues of the body and the routes by which it leaves the spinal theca

Method of assay and anaesthetic employed

The method of assay had to satisfy two main criteria (1) indifference to the nature and amount of the diluting media, (2) a high degree of sensitivity the former since it was proposed to undertake analyses both in body fluids and various tissues and the latter since it was not intended to

employ disproportionate doses of the anaesthetic Of chemical methods the diazo-reaction is probably one of the most sensitive for procaine assay, Kustner and Eissner (1930) and Willstaedt (1934) claimed sensitivities to 0.01 and 0.005 mg respectively for their techniques, while Bullock and Macdonald (1938) asserted that concentrations below 0.02 per cent were beyond their powers of estimation. It was anticipated that the tissue concentration after a normal spinal anaesthetic in the cat would usually be below any of the limits given above, while the gross excess of tissue bespoke laborious separation techniques in which the accumulated error promised to be large even if enough drug were present to render assay possible

A tracer technique seemed, therefore, to be Although theoretically any of the indicated atoms of the procaine molecule is open to substitution by its radioactive counterpart, such a procedure was found impracticable when this research commenced because of limited half life, uncertainty of supply, difficulties of assay, or synthesis with the elements normally present. It was therefore decided to introduce some other element into the benzene nucleus without radically changing the pharmacology of the anaesthetic The halogens were the obvious choice, and preliminary experiments showed that of these Br82 was the most satisfactory Procaine was accordingly brominated by the method of Morel, Leulier, and Denoyel (1929), the necessary hydrobromic acid being prepared as described by Howarth (1948) In this description the author made use of calculated quantities of procaine in the bromination stage but subsequent experience has shown the reaction to be sufficiently easily controlled to render this unnecessary

The method was as follows the concentrated hydrobromic acid having been obtained 3 or 4 cc of 100 vols H.O. were added to it followed by a knife

point of procaine The solution in the tube promptly developed a yellow tinge. Further procaine was then added and the coloration disappeared, to return again after a minute or two when more procaine was added. This procedure was repeated until the reaction was completed. In this way the speed of bromination was increased and the necessity for time-consuming estimations eliminated.

The bromine for these experiments was obtained either as KBr from the cyclotrons of Liverpool or Cambridge or as ethylene dibromide from the small or large pile at Harwell When the small pile was employed 3-6 ampoules each containing 10 cc of ethylene dibromide and 5 per cent aniline (Le Roux ct al 1939, Lu and Sugden, 1939) were irradiated the total activity per ampoule being of the order of 200 millicuries With the large pile (factor of 10) 1-2 cc were irradiated After this the bromine was extracted as KBr by the Szilard-Chalmers technique (1934)

The dibromoprocaine hydrobromide C_{1,}H_{1,}Br₂N₂O, HBr (DBP hydrobromide) thus obtained was converted to DBP hydrochloride either by the method of Morel et al (1929) or latterly by precipitating the base with N/10 NaOH, dissolving the latter in ether, separating and precipitating the hydrochloride with a stream of HCl gas. The entire operation from KBr to DBP hydrochloride occupied about three hours

Assay was carried out by means of a standard Dynatron X 200 scaling unit and a counter designed to take 10 c c of liquid Body fluids were assayed directly tissues were broken down in alcoholic lithium hydroxide (Howarth 1949a) and suitable dilutions of the suspension employed

Preliminary studies

Three preliminary observations were needed

- (1) Investigation of the spinal anaesthetic properties of DBP
- (2) An assessment of the accuracy of the author's method of radioassay
- (3) Study of the fate of brominated benzene rings in the body

(1) DBP as a spinal anaesthetic

Morel et al (1929) in their description of DBP did not include studies of its applications in spinal anaesthesia

The anaesthetic properties of DBP were examined as follows six cats were premedicated with atropine and lightly anaesthetized with ether each received 10 mg DBP, HCl in 1 c c H₂O at 40° C by lumbar puncture and the ether administration was then discontinued

All showed relaxation of the anal sphincter (lower sacral block), loss of knee jerks (lumbar block) but no obliteration of the elbow jerk (lower cervical roots). The hindlimbs were atonic

and could be placed in exaggerated postures On recovering consciousness all the animals appeared paralysed from the lower costal margins downwards, they dragged their hindlimbs They displayed no interest in violent stimuli administered to their hindlimbs, contrary to the effects of similar stimuli to their forelimbs. In one animal there was evidence of some degree of intercostal paralysis as shown by reduced thoracic and increased diaphragmatic excursions animals recovered completely and walked perfectly within 24 hours None showed tail droop after this period. It was concluded that DBP was a powerful spinal anaesthetic but with limited usefulness owing to its low solubility. Although only six cats were examined under these controlled experimental conditions, the author has administered DBP to some 200 cats and six monkeys without producing any persistent ill effects except in four cases, and in at least two of these a persistent paraplegia was due to haematomyelia after lumbar puncture

(2) Accuracy of radioassay

In order to investigate this an anaesthetized cat received 0.5 c c of a DBP, HCl solution by lumbar puncture. Six samples of cerebrospinal fluid were collected and each assayed by the author and by Dr. K. Bullock, who kindly undertook the chemical estimations. The basis of the latter was diazotization and coupling followed by photocolorimetry. Standards prepared gravimetrically were also separately assayed. The results showed a satisfactory agreement (Table I)

TABLE I CONCENTRATION OF DIBROMOPROCAINE HCL IN SUCCESSIVE SAMPLES OF CEREBROSPINAL FLUID (G /100 C C)

Sample Number	Estimation Diazo Dr Bullock	Estimation Radioassay Author	
1 2 3 4 5	0 043 0 037 0 022 0 011 0 005	0 050 0 041 0 024 0 012 0 0041	
Vol of CSF assayed	01cc	0 02 c c	

Estimation of a standard solution (g /100 c c)
Gravimetric 0 015
Diazo assay 0 018
Radioassay 0 016

In later experiments closer agreement has been reached

(3) Fate of DBP

On account of its great stability it is unlikely that DBP suffers removal of its nuclear halogen atoms during its sojourn in the body (Fromherz, 1928, Williams, 1947) Nevertheless, from the point of view of laboratory manipulation, stability of a halogen attached to a benzene ring within an organic compound is influenced by the arrangement of the various groupings about such a ring Thus it was expedient to investigate the drug from this point of view, since the removal of a large proportion of tagged atoms would severely reduce their value as a tracer for this molecule

The method was as follows

An anaesthetized cat received by the preaxial vein of the forelimb 01 g. DBP in 20 cc water, injection being made slowly so as to avoid cardiac or respiratory depression After two hours the animal was sacrificed, the abdomen opened, and the urine aspirated from the bladder, 1 cc of the urine was placed in each of five small tubes. To each of these were added 2 cc of a NaBr solution to serve as a carrier, and to four of them 05 cc of a saturated solution of The precipitate of AgBr was filtered off and washed thoroughly, the filtrate being made up to The figures 10 cc and subjected to radioassay obtained were compared with those derived from the tube containing no AgNO, The results (Table II) showed that most of the bromine atoms in the urine did not exist as ionizable bromine, but as bromine in organic combination The last two lines of the Table indicate that the anaesthetic itself, prepared by the methods described did not contain ionizable bromine

It has been shown recently that even after five days the urinary bromine exists chiefly as bromine in organic form

TABLE II

This table shows that the radioactivity of urine after intravenous dibromoprocaine hydrochloride was not reduced by treatment with sodium bromide (carrier) and silver nitrate followed by filtration. The radioactive bromine atoms have not been precipitated by the silver nitrate, therefore the bromine is organically bound. The last two lines show that there is no ionizable bromine in the anaesthetic itself.

Evn	C	Contents of tube (c c)						
Exp No	Urine	Silver nitrate	Sodium bromide	DBP HCl soln	dardized counts per min			
1 2 3 4 5	1 1 1 1	0 0 5 0 5 0 5 0 5	2 2 2 2 2	0 0 0 0	684 642 651 656 684			
6 7	0 0 Sta	0 5 0 tistical err	2 2 or <u>-</u> 4%	I S D	160 165			

Note on interpretation of results

The concentration of DBP hydrochloride in an aqueous solution was estimated by comparing its counting rate with that of a standard of known concentration. In the tissues, however, procaine and probably DBP is broken down into a number of products (Burgen et al., 1948, Goldberg et al., 1943). In the present work the results of comparison with standard solutions of DBP are expressed as μg of the latter per g (or c c) of tissue. This should be understood as that amount of DBP, HCl which would be present if all the benzene nuclei were incorporated in molecules of DBP.

DISTRIBUTION IN BODY FLUIDS

In this series of experiments it was decided to investigate the levels of DBP obtaining in the CSF, blood, and urine after intrathecal injection

Method

Cats anaesthetized with pentobarbitone received various doses of DBP, HCl by lumbar puncture The needle was left in situ with stilette in position, but at fixed time intervals the stilette was withdrawn, and, after permitting a few drops of CSF to escape, the inside of the butt of the needle was cleaned with a spill of filter-paper and 0 02 c c of CSF was collected in a blood pipette. Blood was taken at similar time intervals from a cannula inserted into the femoral or Urine was obtained by suprapubic carotid artery cannulation of the urethra, the cannula being tied in position and led by way of a narrow rubber tube into a small collecting phial The phial was changed at 15-min intervals, the last of the urine being expressed by gentle pressure over the bladder just before the change of receiver Further specimens of CSF were obtained from the cisterna magna by means of a hypodermic needle passed through the atlantooccipital membrane, the volume removed at each sampling being 01 cc The various specimens obtained were subjected to radioassay

Results

The graph (Fig 1) shows the results obtained from one experiment. The main features are the rapid initial decline of the drug concentration in the CSF, followed by a less steep phase, the rapid rise of urine concentration followed by a slower fall, and the low steady blood level of drug. The concentration within the cisterna was usually so low as to render accurate assay valueless

Five animals were studied in detail and the curves for urine, CSF, and blood found to be similar in form for each animal though there was some individual variation, thus though the decline of the concentration of CSF was always rapid, the actual rate of fall varied. There appeared to be

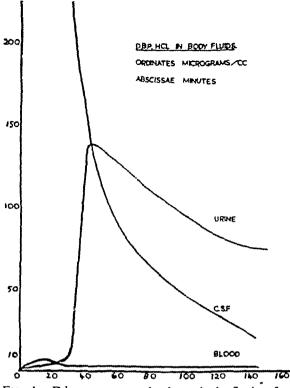


Fig 1 —Dibromoprocaine levels in body fluids after spinal subarachnoid injection

no simple relationship between rate of decrement in concentration, dose of drug, and weight of animal. The "bump on the blood level seemed to correspond to the period of maximum decline of the CSF concentration. The form of the urine curves presented no striking differences except that the peak level varied both as regards time and maximum height.

TISSUE DISTRIBUTION

The observation that DBP rapidly leaves the theca, appears in the urine in high concentration, and maintains a persistently low blood level made it of interest to study the distribution of the drug among the body tissues. A preliminary note of this study has been published (Howarth, 1949b)

Methods

After subaraclinoid injection

The cats were anaesthetized with pentobarbitone the carotid artery cannulated and a spring clamp placed upon the vessel proximally. The bladder was catheterized and a dose of DBP administered by lumbar puncture the needle and stilette being left in situ. The presence of spinal anaesthesia was confirmed and the animals left for a specific period. Fifteen minutes before autopsy the bladder was

emptied and the catheter clipped so as to collect within the bladder a 15-minute specimen of urine Preparation was now made for autopsy, and a sample of blood (1-2 cc) was taken in a small tube and shaken with a few crystals of sodium citrate in order to prevent clotting. The clamp was removed from the catheter and the urine collected. The stilette was withdrawn from the lumbar puncture needle and after allowing two or three drops of CSF to escape, 002 cc of CSF was collected in a blood pipette and discharged with washing into about 4 cc of water

Autopsy

Autopsy was performed in the usual manner, special efforts being made to prevent loss of blood

The spinal cord was removed complete with membranes whilst the animal was still alive, so as to reduce post-mortem absorption of DBP remaining in the cerebospinal fluid. The dura mater was stripped from the spinal cord and the roots of the cauda equina divided close to the cord and retained for assay. One centimetre of cord was removed from the lumbar region at the site of introduction of the DBP. Neither roots nor cord were cleaned, and consequently some of the administered anaesthetic adhered to their surfaces.

Removal of samples

In removal of specimens from an organ adherent blood was removed from its surface and a small section excised, free blood being gently swabbed from the cut surface. An endeavour was made to remove the same portion of each organ in each experiment A list of samples is appended with relevant notes

Cord and roots (see above)

Sciatic nerve in its entirety

Medulla oblongata from the level of the atlas to the lower border of the pons

Spleen about half an inch from its left-hand extremity

Lner A portion from the middle of the anterior surface of the right lobe

Bile aspirated from gall-bladder after liver specimen had been removed

Kidney This was incised along the convexity and the organ decapsulated by pressing it through the incision. The kidney was divested of cortex with a razor blade starting at the attachment of the ureteric pelvis which marks the cortico medullary junction. Cortex and medulla were assayed separately

Muscle The belies of the left hamstrings Lung Lower quarter of the right lung

Gut contents These consisted of the contents of the third part of the duodenum and were extracted by massage of the isolated length of gut held over a watch glass

Gut A portion of the second part of the duodenum

Thyroid and testis (or ovary) in their entirety

Skin A portion from the scalp shaved prior to autopsy

TABLE III
TISSUE/BLOOD RATIOS (Ct/Ca) AFTER INTRATHECAL INJECTION OF DIBROMOPROCAINE HYDROCHLORIDE

Weight of cat (kg) Dose (g) Time after injection (hr)	3 6	2	3 5	2 1	3	2 5	2 25
	0 007	0 007	0 025	0 007	0 008	0 03	0 005
	1	1 23	2 5	3	21	39	20
Blood* Spleen Medulla Sciatic nerve Muscle Lung Skin Thyroid Testis Gut Kidney cortex Kidney medulla Liver Bile Urine Cord* Roots* C S F * Duodenal contents* Error ±	3 36 0 26 0 091 0 27 0 11 0 73 0 5 0 49 0 53 0 34 8 43 6 4 2 3 6 73 83 38 3 163 103 6 76 3 4%	5 41 0 25 0 11 0 37 0 1 0 52 0 32 0 27 1 5 3 1 9 3 4 3 2 8 26 6 27 26 94 37 92	3 34 0 24 0 074 0 2 0 049 0 47 0 31 0 19 0 28 0 48 6 2 2 6 1 7 7 2 24 26 76 19 7 7 2	2 73 0 30 0 13 0 48 0 067 0 67 0 63 0 4 0 54 0 56 7 2 2 7 1 4 8 6 133 8 4 76 22 18 3	1 31 0 50 0 30 0 84 0 13 0 80 0 93 0 80 0 6 1 3 1 5 1 1 9 8 4 0 64 1 6 1 3 None	2 86 0.22 0 12 0 31 0 074 0 44 0 58 0 30 0 29 0 36 0 88 0 34 0 67 0 8 0 6 0 8 1 7 3 6	0 78 2 51 0 25 0 63 0 19 0.50 1 29 0 59 3 40 1 80 1 35 5 7 15 7 0 55 0 87

^{*} Expressed as µg /g

Estimation

Specimens were placed on watch glasses on a tray over which a wet cloth was suspended in order to reduce evaporation. The specimens were weighed within a few minutes of removal from the body, the smaller organs—i e, thyroid, nerve roots, and ovary—being weighed immediately. Assay was undertaken as described above

Results

Table III shows the results obtained from seven animals. The drug concentrations found in CSF cord, roots, and duodenal contents were not directly dependent upon blood concentration, and these were expressed as $\mu g/cc$ or $\mu g/g$ of tissue. The rest of the tissue concentrations were expressed as the ratio between mass of drug per gramme of tissue and mass of drug per cc of blood (Ct/Ca) ratio

The blood contained in the vascular bed of the organ under consideration could not be satisfactorily removed. Thus each assay included an unknown modicum of drug in the blood of its vascular bed. This capillary volume could not be expected to remain constant in different animals. Furthermore, the doses of drug and the time intervals before autopsy were also varied, and hence consistent results were not anticipated. This difficulty has been mentioned by Wallace and Brodie

(1937) in their estimations of tissue iodides and thiocyanates

Of all the tissues examined, apart from those in direct contact with the drug in the CSF, only liver and kidney showed a tissue concentration above that obtaining in the blood (tissue/blood ratio) Of these the kidney values were much the higher, and in the majority of the assays the cortex showed a higher concentration than the medulla The concentration in the duodenal wall appeared puzzling and erratic until it was found that the bile contained an appreciable amount of DBP It is interesting to note that both blood and CSF contained detectable amounts of DBP 39 hours after its injection

The concentrations found in the cord and roots show special features. In all but one experiment the drug concentration was lower in the cord than in the CSF, but the roots always contained a higher concentration of drug than the cord. It appeared that the roots and not the cord were capable of concentrating the drug from the CSF in which they were bathed

The medulla oblongata showed a very constant tissue/blood ratio, and such constancy would not be expected had the drug ascended within the spinal subarachnoid space to any significant extent. The tissue distribution after intravenous

TABLE IV TISSUE/BLOOD RATIOS (Ct/Ca) AFTER INTRAVENOUS ADMINISTRATION OF DIBROMOPROCAINE HYDROCHLORIDE

		Values f	or Ct/Ca after	r intravenous	injection	
Weight of cat (kg) Dose (g) Time after injection (hr)	2 5 0 036 1 20	3 75 0 17 70	2 5 0 092 4	2 6 0 05 2	2 5 0 078 4	3 0 12 2
Spleen (0 25) Medulla (0 11) Sciatic nerve (0 33) Muscle (0 08)	0 27 0 14 0 4 0 14	0 49 0 19 0 5 0 11	0 28 0 14 0 33 0 11	0 2 0 12 0 38 0 09	0 18	0 1
Lung (0 57) Skin (0 47) Thyroid (0 34) Testis (0 41) Gut	0 81 0 53 0 34 0 78 0 93	0 75 0 87 0 65 0 31	0 71 0 30 0 53 0 71 0 08	0 4 0 28 0 46 0 55	0 6 0 57 0 5	0 55
Kidney Liver Bile Urine	5 75 2 6 None 39	1 23 0 38 0 66 0 27	1 71 0 32 25 6 30 2	2 4 1 23 6 02 30 6	1 06	1 46
Cord Roots Blood (mean level 3 54 µg /g)	0 14 0 27 22 1	0 17 0 44 4 52	0 05 0 24 33	0 045 0 22 43 9	0 12 0 41 11 4	0 06 0 26 38 8

Figures in parentheses are mean Ct/Ca ratios after intrathecal injection

injection was also studied in order to ascertain whether any difference existed between root and cord concentrations, and to study the effects on tissue/blood ratios of higher blood levels

The drug was injected into the preaxial vein of a forelimb and assays of organs made at various The results are tabulated (Table IV) It will be noted that four animals were studied in detail, but that estimations were made of tissue/ blood ratios for cord and roots in six animals For convenience of reference the mean tissue/ blood ratios for the intrathecal analyses are included in the left-hand column In this series of means, the 21-hour-period results are not included, means have not been provided for gut, bile, liver, kidney, or urine, since these results were variable, depending largely upon the state of the animal and the time interval between administration and sampling In a similar way no means are tabulated for cord and roots since these values are mainly dependent on the concentration of drug in the theca

The results show that even after intravenous injection the roots reveal a higher concentration of drug than the cord. On the whole the tissue/blood ratios after intravenous injection show a slight general increase, but in view of the relatively enormous blood levels it is doubtful if this increase has any real significance.

The concentration of the drug in the contents of the descending colon was assayed and also the concentration obtaining in the bone marrow of the femur. The values were of the order 2 μ g/g and 0 23-0 34 (tissue/blood ratio) respectively

Only two observations were made and no assays were undertaken of the entire mass of faeces the results merely indicated that some of the drug was excreted via the gut

The concentration of drug in the cisterna magna was usually insignificant except in one case where the concentration rose to $1.9 \, \mu g / c \, c$ after $1\frac{1}{2}$ hours. It was of interest, since assays of the cervical cord were planned, to discover the origin of this drug in the cisterna, some had undoubtedly risen from the lumbar regions, but it was possible that some had been secreted by the choroid plexuses

DBP and the blood/CSF barrier

DBP was administered by the preaxial vein of the forelimb, and specimens of CSF were taken at specific intervals from the cisterna magna Blood samples were taken at similar periods from the right femoral vein. A graph of such an experiment (Fig. 2) shows that DBP circulating in the blood in sufficient concentration was able to pass the choroid plexus.

Since it had been ascertained that DBP passes rapidly into the blood stream and its distribution

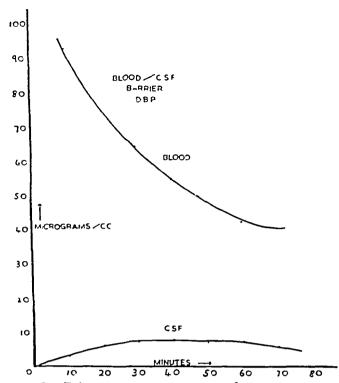


Fig 2—Dibromoprocaine appears in low concentration in the cerebrospinal fluid after intravenous injection of 132,400 μg of DBP, HCl (Cat 2 5 kg)

among a number of organs had been studied, it became of interest to investigate the route by which it left the spinal theca

The possibility of lymphatic drainage will first be considered

Lymphatic drainage of the subarachnoid space

Since the lymphatics draining the region of the

lumbar theca ultimately enter the thoracic duct, it was decided to cannulate this duct above the diaphragm This was effected in the following manner Under nembutal anaesthesia the lower part of the thoracic aorta was exposed by the appropriate nb resection, haemostasis being obtained by previous ligation of the ribs in front and behind the proposed resection and afterwards by diathermy When the thorax was opened the animal was maintained with artificial respiration and the lungs were packed away from the aorta The exposure was extended caudally into the costophrenic angle, and by depressing the cupola of the diaphragm a view of the aortic orifice was obtained. The parietal pleura was dissected away from the right side of the aorta until the thoracic duct appeared like a

white thread on the pink aortic wall. The duct was ligated and operations discontinued for 30 minutes

On recommencing the operation, the distended cisterna chyli could be seen at the aortic orifice The pleura was removed from the cisterna chyli and a loose ligature cast around the upper part of the latter A large-bore, curved, and blunted intramuscular needle was used as a cannula, and this was driven by steady pressure into the cephalic end of the cistern and tied in position A male connector bearing a short length of cycle valve tubing was fixed into the cannula and the wound closed so as to leave an inch of tubing protruding through the chest wall The chyle flowed freely at first, but the cannula frequently became It was then necessary to empty the obstructed cistern by gentle aspiration with a syringe shows the exposure and cannulation of the thoracic duct

Introduction of DBP

The muscles of the back are drained in part by the lymphatic channels which enter the thoracic duct (Gray, 1946), and introduction by lumbar puncture was thus unsuitable as a routine procedure since leakage into the muscles of the back was possible, in order to avoid this the theca was exposed where it passes over the sacral promontory and a narrow-bore cannula introduced into the subarachnoid space and tied in position

Samples of chyle and CSF were taken at frequent intervals after introduction of DBP, HCl Since the maximal fall of CSF concentration occurred over the first 30 minutes (Fig 1) it was decided to limit the observations to this period

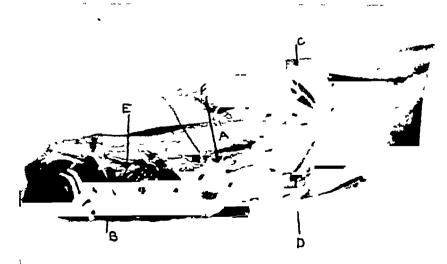


Fig 3—Supra-diaphragmatic cannulation of thoracic duct. The chest has been opened A, aorta, B, intercostal artery, C, crus of diaphragm, D, splanchnic nerves. E, curved cannula and rubber connector, F, tip of cannula in thoracic duct.

Results

Fig 4 shows that the CSF concentration fell rapidly notwithstanding the new method of introduction and the extensive surgery All six animals thus investigated showed this. The quantity of

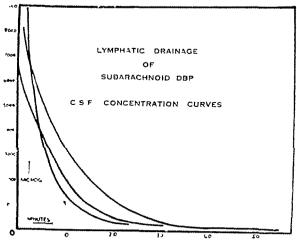


FIG 4—The rate of fall of the concentration of dibromoprocaine in the cerebrospinal fluid is rapid despite cannulation of the thoracic duct. The concentration of drug in the duct is too small to show on this scale

drug excreted by the thoracic duct was in all experiments too low to be represented on the graph. Table V gives the figures for the experiment relating to curve 2 on Fig. 4. It is evident that the total excretion by the thoracic duct is not a considerable factor in the reduction of drug concentration in CSF.

In a comparable animal receiving the same dose but administered by lumbar puncture, the total

TABLE V
CAT 624 kg dose by sacral cannula 12000
MICROGRAMMES

Time after	DBP in	Chyle		
injection (min)	CSF (µg percc)	Volume (c c)	DBP (μg)	
1	7,800	0 40	0 37	
4	5,500	0 30	0 27	
8	4,300	0 32	0 26	
12	2 900	0.50	0 44	
16	1,700	0 40	0 32	
21	1 100	0 35	0 63	
33	260	0.50	1 14	
55	160	0 25	0 31	

Total volume of chyle = 3 02 c c Total excretion = 4 24 μ g = 0 035% of total dose drug excretion in the chyle attained 243 μ g in 27 minutes, during which time the CSF concentration fell from 8.620 to 245 μ g /cc

Passage of DBP into the spinal cord

The possibility of absorption of drug by the intrathecal neural elements was next considered Previously (Table III) it has been shown that the roots appear to concentrate the drug from the CSF but that the cord does not There is no information about the concentration within the cord substance itself, and the values obtained include an indeterminate quantity of anaesthetic clinging to the surface. The technique first employed was that of radio-autography

Method

It is a sine qua non in radio autography that the position of the active substance in the specimen must not change as a result of the application of histological reagents, but though the final form of DBP in the nervous tissues was unknown the difficulties inherent in the solubility of DBP, HCl itself were fully realized. A rapid fixation of the drug in the tissues was necessary to ensure that diffusion would not occur after death

In an anaesthetized cat laminectomy was performed over the lumbar enlargement The dura was held The C.S.F was allowed apart by small haemostats to escape and the knee jerks elicited. A pledget of wool soaked in 05 per cent (w/v) DBP, HCl in water or Dale's Ringer at body temperature was placed on the lumbar enlargement, absent knee jerks indicating the onset of anaesthesia. The pledget was replaced every five minutes until five changes had been made Five minutes after the last application the spinal cord was clamped above and below the site of medication and the intervening length of cord with its attached roots removed en masse from the vertebral canal The cord was severed just beyond the site previously occupied by the wool, and using the upper clamp as a handle the remainder of the cord was plunged forth with into a Dewar flask containing liquid air cord was frozen solid and the anaesthetized portion isolated with an ampoule saw placed upon the stage of a freezing microtome, and sections of 30 μ were The sections were laid in the centre of small cells constructed upon coverships and flattened with a The coverslips were placed immediately on a metal plate kept "cold" by liquid air The cover slips were then transferred to small squares of Ilford no-screen v-ray film, resting upon small circles of Each cell was placed in a typewriter thicker glass ribbon box and stored in the refrigerator for a week A specimen was inspected from time to time to ascertain that it was still frozen to develop a film and to inspect the autograph produced at various time intervals Subsequently all were developed and the sections returned to the refrigerator during the

development period when the coverslips bearing the sections were superimposed on the negative and the area of darkening in the latter ascribed to the relevant parts of the cord

Figs 5A, B, and C are autographs obtained by this technique. They exhibit a zone of radioactive anaesthetic surrounding and outlining the periphery of the cord. Within this zone are the nerve roots cut in transverse section, and to demonstrate

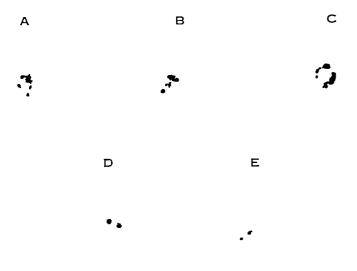


Fig 5—Radioactive anaesthetic around a number of cord sections In D and E most of the anaesthetic has been washed away, but that in the roots remains These autographs were made by a crude technique before the advances of Bélanger and Leblond (1946) and Pelc (1947) For details see text

them a number of sections were rinsed in water before placing them on the coverslips Figs 5D and E show the results of this manœuvre, the roots containing a higher concentration than the cord and revealing themselves as intense white spots

Results of these autographic studies should be interpreted with caution since the edge of the cord could not be related to the edge of the blackened area exactly, and the apparent absence of drug in the centre of the cord may simply indicate a concentration inadequate to affect the film (Hamilton 1942)

Direct estimation of penetration

It was desirable to determine by some more sensitive method whether any drug had penetrated into the depths of the cord

Method

In a number of anaesthetized cats, cannulae were inserted into the right common carotid artery and the sacral subarachnoid space. A dose of DBP was intro-

duced by the sacral cannula A hypodermic needle was passed into the cisterna magna. After a specific interval a specimen of blood was taken from the carotid artery and samples of CSF from both the cisterna and the lumbar sac. After laminectomy the cord and membranes were removed from the upper cervical to the lower sacral, region bleeding being controlled by diathermy and haemostats. The cervical end of the cord was held in artery forceps and the entire cord lowered slowly into a Dewar flask con-

taining liquid air The frozen cervical and lumbar enlargements were isolated Specimens were now removed from the centre of the lumbar enlargement The cervical portion was returned to the liquid air to await The lumbar enlargement, wrapped in gauze and lint, was permitted to thaw until it reached a consistency suitable for cutting wrapped piece of cord was held in the hand and the upper half-inch of dura was incised longitudinally on the ventral and dorsal aspects, the two flaps so formed being reflected over the rest of the cord by retraction with toothed forceps The underlying pia was heavily contaminated with radioactive DBP, and it was essential to avoid contact between this and the tissue

The pia and periphery of the cord were removed by making thin longitudinal tangential slices all round in such a way that the ends of the slices overlapped The knife was washed

and dried before each cut and the cord returned to the liquid air from time to time in order to maintain its frozen state. In this way a central core of relatively uncontaminated cord was obtained which was severed near the projecting uncut cord

A chrome steel cylinder having at one end a razorsharp cutting edge had been prepared and was placed with its cutting edge against the uncontaminated cord By gentle screwing movements the cylinder was driven through When a suitable distance had been traversed the cord was incised around the metal cylinder just proximal to its cutting edge. The rest of the cord was gently pulled away from the severed collar until a portion of the cord centre cut by the metal cylinder became visible This was then cut through central part of the cord contained within the cylinder was now driven out a few millimetres by pressure from a metal rod The end of the protruding cord was cut off and the rest driven out entirely, the other end of the cord also being removed The remaining rim of cord tissue was inspected to make sure that the cutter had not penetrated the contaminated pial covering order to establish this the entire rim of cord tissue was placed in formalin, sectioned serially, and examined microscopically (Fig. 6). Alternatively the





Fig 6



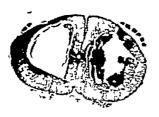


Fig. 7

Figs 6 AND 7 —Sections of the remaining cord tissue after removal of the deeper portions (6) with a cylindrical cutter, (7) with an iris knife. In all sections the peripheral portions are intact.

rim was bisected transversely and the upper portion assayed and the lower sectioned as above

The central rim of the cord was then weighed broken down in lithium hydroxide, and radioassayed The cervical cord was assayed in a similar manner

In some of the sections it was found that the tip of the anterior median fissure was included in the removed cord centre. It is improbable that DBP entered the fissure in any significant amount since it was not outlined in the autographs.

order to eliminate this, after the previously described precautions had been taken, a small portion of each lateral white column of the spinal cord was removed with an iris knife and assayed, sections being made of the peripheral rim as before Examples of such sections are shown in Fig 7 It will be seen that the anterior median fissure has not been encroached upon The results of assay were comparable with those of former experiments

Results

The results (Table VI) show that the blood concentration of drug is high enough to be easily assayed in five minutes after subarachnoid injection. There is a possibility, therefore, that the tissue concentrations obtained are due to the blood in their capillary beds rather than to DBP which has directly penetrated the cord. Studies have already been made of the tissue/blood ratios in respect of the cord after intravenous administration of the drug. The observed results of the cord centre concentration are similarly expressed, and it will be seen from Table VI that the ratios cal-

culated for this series of experiments are far higher for both the cervical and the lumbar cord centres than the corresponding ratios after intravenous injection DBP has entered the cord by some route other than the blood stream, presumably through the pial membrane in the case of the lumbar cord

The results recorded for the cervical cord are of special interest, for here it can be seen that the

TABLE VI

DISTRIBUTION OF DIBROMOPROCAINE HYDROCHLORIDE WITHIN THE INTRATHECAL NEURAL ELEMENTS

All values expressed as $\mu g/g$ or $\mu g/c$ c unless otherwise stated

Time (min) Dose (g)	5	10	20	20	25	25
	0 012	0 0033	0 0033	0 0033	0 0033	0 012
Roots CSF lumbar Cord periphery lumbar Cord centre lumbar Cord periphery cervical Cord centre cervical CSF cervical Blood level Cord centre lumbar Ct/Ca Cord centre cervical Ct/Ca	1,000	320	246	355	63 7	400
	3,900	1,340	106	190	273	203
	520	138	66	138	30 9	300
	120	53	2 6	5 8	18	49
	17	05	0 42	0 7	6 5	23
	7	07	2	0 8	0 5	19
	3	±00*	±00*	4 00*	5 4	09
	2 9	0 646	0 692	0 78	0 877	27
	41 4	8 21	3 76	7 44	20 5	18 1
	2 41	1 08	2 39	1 03	0 57	0 705

^{±00 =} Too small to estimate Ct/Ca cord ascertained in previous experiment (Tables III and IV) always less than 0.2.

concentration in the cord periphery is higher than that in the CSF which bathes it There are three Either the drug possible explanations for this has ascended from the lumbar regions via the pial plexus of veins, or by the neural elements within the cord, or the cervical cord has specifically absorbed and concentrated the drug locally from the CSF Specific concentration is unlikely in that it is not a feature of the behaviour of the cord in contradistinction to the roots (Table III) Spread within the cord is likely, since Brierley and Field (1948) have shown that phosphorus injected into the sciatic nerve can be found in the upper reaches This passage upwards in the of the spinal cord deeper parts of the cord is probably not within the deep veins, since these drain transversely into a superficial longitudinal venous plexus (Mettler, 1942) and do not travel the length of the cord within its substance. It is the results obtained for the cervical cord centres that make some degree of cord penetration by a spinal anaesthetic reasonably certain

It will be seen that tissue/blood ratios for cord centre and cord periphery have been compared with tissue/blood ratios established for the entire cord. It is possible that tissue/blood ratios for cord centre and cord periphery differ. In order to elucidate this a monkey received an intravenous dose of DBP and the ratios were ascertained for both the central and peripheral parts of the spinal cord. They proved to be 0.21 and 0.23 respectively

It must be emphasized that it is not possible to compare results obtained from different animals since cord diameters vary and the cutter did not Consequently a central core removed from a cord of small diameter will encroach further upon the periphery than a similar core removed from a cord of large diameter, and it is in these peripheral portions that a higher concentration of anaesthetic is to be expected

Note on fixation

Neural fixation has been stated to be responsible for the decrement of drug concentration in the CSF further it has been claimed (Stout, 1929) that such fixation prevents ascent of the drug to the vital centres. This is untenable. It has never been suggested that sodium phosphorus, or bromine suffer such fixation, none the less, compounds of these elements do not appear within the cephalic reaches of the spinal subarachnoid space in any significant amount after intrathecal injection. Further, the rapid appearance of these substances in the venous system clearly points to a

more likely mechanism (Howarth and Cooper, 1949)

Of the neural elements within the theca only the spinal roots show regularly a capacity to concentrate a spinal anaesthetic to a level above that in the CSF, to this phenomenon alone is the term "fixation" applied with precision

"Fixation" implies retention, though this may occur, the total quantity of drug involved must be small since there is no appreciable arrest of the drug within the theca, the anaesthetic appearing almost at once in the venous drainage of the part. In the decrement curves there is no striking difference between those of DBP, sodium, phosphorus, and bromine, and there is no reason to believe that any of the latter three substances is retained within the theca (Howarth and Cooper, 1949) Even in the roots the total amount of DBP retained must be small

The problem of neural block by the anaesthetic within the cord

In the last section it was shown that DBP could penetrate the spinal cord, and it was of interest to discover whether the concentration there was adequate to block the pathways within the cord. The respiratory pathway was selected for study. According to Starling's textbook (1936) this pathway conveying impulses from the respiratory centres to the cells of origin of the intercostal nerves is situated in the deeper portions of the lateral columns of the cord. Pitts (1940) localized this pathway in the anterior and antero-lateral columns of the cord. Accordingly it was decided to apply an anaesthetic to the cord in an attempt to block this pathway.

Method

In all 45 cats and four monkeys were examined Laminectomy was performed on the anaesthetized animal extending from the 6th cervical to the 2nd or 3rd thoracic vertebra Bleeding was controlled and the dura incised along the midline between the caudal and cephalic extremities of the wound flaps were removed until only a small band of the membrane remained on the ventral aspect of the A swab moistened with warm Dale's Ringer was placed in the wound and a cannula was inserted into the femoral artery and connected to a bloodpressure apparatus Tambours were placed on the middle of the thoracic wall and upon the anterolateral abdominal parietes so that thoracic and abdominal respirations could be recorded simultaneously

The animal was placed upon a board with its caudal end elevated through an angle of 60° the animal s

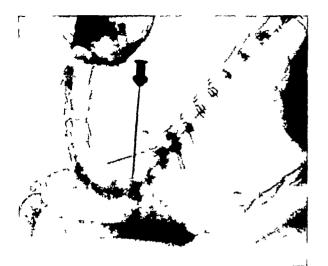


FIG 8 —Skeletal relationships before application of anaesthetic to cervical cord. Needle indicates the middle of a three-segment laminectomy

head raised by means of a clamp fixed to its lower jaw and the extension of the neck continued to the limit of its free movement

Thus the lower cervical part of the cord was placed in the depths of the wound at the lowest part of the U (Fig 8) In this way spread of the anaesthetic placed in the base of the U was reduced, sparing the phrenic roots proximally and the intercostal nerves distally

The smoked drum was set in motion and after a short interval a pledget of wool bearing a warm solution of anaesthetic was placed on the cord and replaced by a further pledget in five minutes. This was repeated throughout the experiment care being taken to ensure that the anaesthetic had access to the entire periphery of the cord.

Results

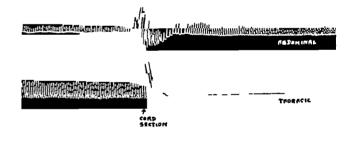
The results were consistent and no interruption of the tract was observed after the application of DBP, procaine, cinchocaine, amethocaine, or cocaine itself Fig 9 shows the end of such a trace after application of amethocaine followed by percaine

Before a satisfactory interpretation could be attempted it was essential to discover if a spinal anaesthetic could produce block of the pathways in the cord even if it were placed in contact with them. Thus a small volume of dilute procaine was injected directly into the cord with a fine needle after first making a small incision in the pia. Under these conditions block of the

respiratory pathway was produced with 02 c c of a 05 per cent (w/v) procaine solution (Fig 10) Neither saline nor water produced such an effect In Fig 11 the phrenic roots were paralysed after cinchocaine at T1 by lowering the head, and there was a compensatory increase in thoracic respiration. Later the respiratory centre was paralysed and all respirat on ceased. Lowering the thorax did not regularly produce intercostal paralysis, and it was necessary to inject the drug under the free edge of the dura into the thoracic subarachnoid space.

Venous drainage of the spinal subarachnoid space

Various routes whereby a spinal anaesthetic may leave the subarachnoid space have been considered, but none has shown itself in an unequivocal manner to be pre-eminent. Since the appearance of the drug in the blood stream is almost immediate (Howarth and Cooper, 1949) it seemed that a direct venous drainage was the most likely route of departure for a substance administered intrathecally Further studies have in a large measure confirmed this belief (Howarth and Cooper, 1949). It has



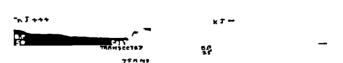


FIG 9—Monkey & 7.5 kg Nembutal, 40 mg/kg IV I per cent amethocaine was first applied to the cervical cord After 50 min 5 per cent nupercaine was substituted 75 min after the original application the respiratory pathway was still conducting Section of the cord at the level of application produced cessation of thoracic respiration and abdominal increase The knee jerks were obliterated The fall of blood pressure is of doubtful significance, the author has observed such a fall without the application of any anaesthetic with the animal placed in the neck-extended position

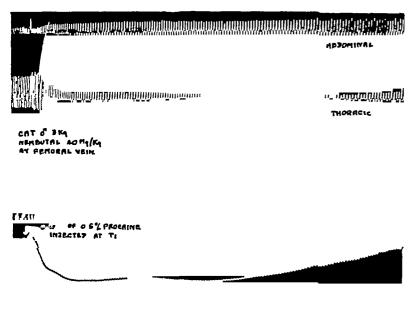


Fig 10—Procaine injected into the spinal cord Interruption of descending respiratory pathway with 0.5 per cent procaine A similar result may be obtained with a much less volume of procaine (0.05 c c of 2 per cent)

been established that the azygos vein is an important channel for the venous drainage of the spinal theca in the cat not only for DBP but also for compounds of sodium, phosphorus, and bromine

DISCUSSION

From the investigations here recorded the following conclusions appear justifiable

After introduction into the spinal theca, the concentrations of DBP, sodium, phosphorus, and bromine show a rapid reduction owing to their departure by certain channels. The substances were found in the blood stream as soon as the injection was completed (Howarth and Cooper, 1949)

As regards the anaesthetic, some is absorbed by the spinal roots which usually contain a higher concentration than that present in the CSF The spinal cord too is peneirated by the anaesthetic, though the concentration within it is relatively small and is inadequate to cause a functional transection of the cord. It must not be assumed that this anaesthetic occup es the perivascular spaces, for although Weed (1914, 1923) has shown that these spaces are in direct communication with the general subarachnoid space, it is probable that the direction of fluid flow is centrifugal rather than centripetal (Weed 1914) Furthermore from

King's work (1939) it appears that dyes gaining access to the cord from the subarachnoid space do so by a general permeation of the pial membrane rather than by local perivascular ingress specific concentration in the supports the contention of Tuffier and Hallion (1900) that the site of action of a spinal anaesthetic is centred upon the nerve roots, a conclusion supported by Pitres and Abadie (1901) Smith and Porter (1915) assumed that the pathways of the spinal cord are interrupted, though Babcock (1925) reverted to the earlier view in accordance with the opinion of Babcock did imply that Labat (1923) the cord was slightly affected, but Graffagnino (1926-7) denied even this degree of involvement, a view endorsed by Campbell (1926), Schutz (1928), and Evans (1929) Subsequently, with the notable exception of Sebrechts (1934), there appeared a reversal of opinion by

the majority of workers Thus Koster and Kasman (1929), Russell (1929), Ferguson and North (1932), Grodinsky and Baker (1933), and Vehrs (1934) concluded that the cord is in fact penetrated by the drug Hill and Macdonald in 1935 stated that spinal anaesthesia was generally accepted to be a root anaesthesia, but Henderson (1937) still

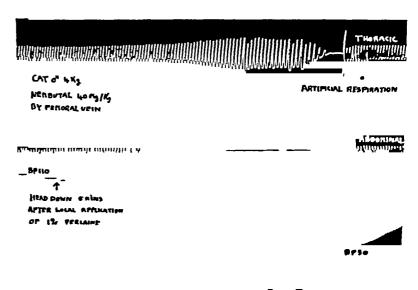


Fig 11—Local application 1 per cent cinchocaine to cervical cord Head down after five minutes. Fall of BP obliteration of abdominal respiration (phrenic roots) compensatory thoracic increase followed by respiratory paralysis, probably owing to arrested activity of respiratory centres. Artificial respiration, BP commences to rise

adhered to anaesthetic cord block Romberger (1941, 1943) restored the status quo ante, holding that the pia mater denies the drug access to the This confusion seems to have arisen from the assumption that an anaesthetic present in the cord must necessarily interrupt the tracts therein So far as the author is aware a quantitative assay of the drug within the cord has not been reported previously, and it appears that only Harrison and Frank (1932) and Kunlin (1945) have blocked the cord by direct injections of anaesthetic

It was evident that no study of the causes of cord sequelae of spinal anaesthesia could be undertaken when the basic facts were so ill-understood The author does not suggest, however, that some of the peripheral tracts are not blocked during spinal anaesthesia, and the fine fibres of the spinothalamic pathway may be interrupted, though he has been unable to devise a satisfactory method for the study of this phenomenon

Some of the drug appears to ascend within the cord to the cervical regions, a route which has been suggested by Brierley and Field (1948) for phosphorus, and some may pass upwards via the pial venous plexus, little passes down the sciatic nerves

A small quantity leaves the subarachnoid space via the lymphatic drainage, thus confirming the presence of the channel suggested by Field and Brierley (1948a and b) in their work with indian ink

From the blood stream the drug is distributed about the body tissues, but only kidney and liver are able to concentrate it above the blood level, the kidney being far more important than the liver in this respect. The tissue/blood concentrations do not appear to show any significant variations with large changes in blood level and the time interval before autopsy It would thus appear that tissue retention does not occur except possibly in the 20-30-hour range

The urine provides the main pathway of removal from the body, and the concentration of the anaesthetic or its end-products is much in excess of the corresponding blood levels, a fact elicited by Burgen and Keele (1948) The blood levels themselves are consistently low The bile forms a subsidiary excretion channel, and, though some of the products may be reabsorbed by the gut, some appear in the faeces Some part of the drug containing benzene nuclei persists for considerable periods in the blood and blood DBP can pass the choroid plexus. Of all the channels draining the spinal subarachnoid space, the venous route

appears to be the most important (Howarth and Cooper, 1949) This is not clear in contemporary teaching

SUMMARY

- A radioactive spinal anaesthetic dibromoprocaine (DBP) hydrochloride has been prepared
- 2 The spinal anaesthetic properties of this drug have been investigated
- 3 The distribution of the drug about the body fluids after its intrathecal injection has been studied It has been shown that the concentration in the spinal subarachnoid space rapidly declines, associated with a rapid rise in urine concentration The blood level remains persistently low
- 4 The tissue distribution of DBP after both intrathecal and intravenous injections has been studied After intrathecal injections only the spinal roots regularly show any capacity to concentrate the drug above the level existing in the cerebrospinal fluid at the site of injection. Of the tissues examined only kidney (and urine), liver (and bile) appear able to concentrate DBP above the circulating blood concentration Large variations in time and dose did not produce large changes in the tissue/blood ratios
- 5 It has been shown that DBP enters the spinal cord during spinal anaesthesia, though it is improbable that it is able to produce a functional cord transection
- 6 Various routes of departure of this anaesthetic from the spinal theca have been studied, and, of these, that furnished by the venous system appears in the cat to be the most important

The author wishes to thank Professor A D Macdonald for facilities in his laboratory and for the benefit of his advice Also Mr Robert West, now of AERE Harwell for the trouble taken in the preparation of many radioactive samples are due also to the Medical Research Council which bore much of the financial burden of this work

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THE EFFECT OF DIMERCAPROL ON LEAD POISONING IN MICE*

ВY

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The published investigations of the effect of dimercaprol or 2 3-dimercaptopropanol (BAL) in lead poisoning in animals have been chiefly concerned with acute poisoning or with toxicity Braun, Lusky, and Calvery (1946), Graham and Hood (1948), and also Germuth and Eagle (1948) reported that dimercaprol failed to protect animals poisoned with lead acetate, and that the dimercaprol-treated animals died sooner In acute poisoning, Ginsberg and Weatherall (1948) found that dimercaprol increased the urinary excretion of lead, and altered the distribution in the organs The present work was designed to estimate the effect of dimercaprol on the retention of lead in a more chronic form of plumbism

METHODS

The mouse was chosen as the experimental animal because the whole animal could be ashed and a determination of the total lead content made Lead acetate was fed with a low calcium diet to male mice in such amounts that the maximum possible daily dose was 1 mg Pb per mouse The procedure followed was similar to that used by Tompsett (1939), who showed that retention of lead in mice was highest The lead acetate in most on a low calcium diet experiments was marked with a tracer of Pb210 (1e, radium D) This naturally occurring isotope has the great advantage of being easily obtained from old radon tubes The lead was fed for 8 to 14 days, and after varying intervals of time the animals were killed, the stomach and intestine removed and discarded, to remove any lead excreted into the gut, and the whole carcass ashed, the ash was brought into solution with the minimum of acid, and made up to 100 ml with water Lead was then estimated chemically in aliquots of the ash solution by the method described previously (Tompsett and Anderson, 1935) Where radioactive lead had been administered, the activity of the ash solution was estimated by means of a Geiger-Müller counter In practice the β radiation of

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radium D is too weak to count and the stronger β radiation of radium E in equilibrium with radium D is counted. Ash samples had therefore to be kept until the mixture reached equilibrium, a matter of 30-40 days

Dimercaprol dissolved in oil, or as a freshly prepared solution in saline, was injected subcutaneously in daily doses of 50 mg/kg mouse, either during the entire period of administration of lead or in the subsequent period

RESULTS

The results obtained when dimercaprol was given simultaneously with the lead are shown in Table I The first two experimental animals

TABLE I

ADMINISTRATION OF LEAD AND DIMERCAPROL (BAL)
SIMULTANEOUSLY

Lead content of mice in mg /100 g a by count b by chemical analysis

Con	trols	Dimercaptol				
<i>a</i> 1 0	b 1 08	a 0 39	b 0 31	Lead 10		
0 96	1 20	0 63	0 56	BAL in oil 8 days		
0 75	0 73	0 48	0 59	Lead 14 days		
1 06 [1.92]	1 11 0 89	0 71 0 40	0 55 0 42	BAL in sa- line 11 days		
Mean 0 94 Range S D	1 00 0 73–1 11 ±0 19	0 52	0 48 0 31-0 59 ±0 12			

received dimercaprol in oil solution daily for eight days, the other three dimercaprol in saline for eleven days. The results of the chemical analysis show that the lead content of the mice receiving dimercaprol was significantly lower than that of the controls. There is not a very close agreement

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between the figures obtained by count and those by chemical analysis, but they are of the same order and serve as a useful check. For some unexplained reason one of the controls, in brackets, gave a very high count, and has been excluded from the calculation of the mean. The lead content is given here as mg/100 g, but as the dead weights were all of the same order the same conclusions are reached if one compares mg of lead per mouse

The lead given was approaching a lethal dose, as in some similar experiments one or more of the animals died during administration of the lead. When mice which had been fed lead previously were treated with dimercaprol the results shown in Table II were obtained. The first two experi-

TABLE II

ADMINISTRATION OF LEAD FIRST THEN DIMERCAPROL (BAL)

Lead content of mice in mg /100 g $\,a$ by count $\,b$ by chemical analysis

Controls		Dimercaprol				
<i>a</i> 1 58	0 72	<i>a</i> 1 17	0 92	Lead 8 days then 7 days		
0 98	1 08	0 89	1 04	BAL in oil last 4 days		
Mean	0 90		0 98			
	0 53 0 95 (20) 0 41 0 45 — (12)		1 15 (17) 0 95 (21) 0 85 (20) 0 50 (22) 0 61 0 47	Lead 10 days then BAL in saline 12 days		
Mean	0 58	' 	0 75			

Numbers in parentheses indicate day of death

mental animals received dimercaprol in oil, and the next six dimercaprol in saline This second series were fed non-radioactive lead. Three of the control animals and four of the treated died near the end of the experiment on the days shown Two animals died before dimercaprol treatment was started and were not analysed Treatment with dimercaprol had no significant effect on the final lead content The figures for the second series show a wide variation owing to the variation in the dead weights in this series where older animals were used (the previous animals were all "20 g mice") If the actual amounts of lead found in the individual mice, as shown in

TABLE III LEAD FIRST THEN DIMERCAPROL

Actual lead content of mice in mg from Table II
Chemical analysis

Controls	Dimercaprol	
0 18	0 23	Lead 8 days then 7 days
0 26	0 27	BAL last 4
0 18 0 19 0 16 0 18	0 22 0 24 0 21 0 15 0 19 0 16	Lead 10 days then BAL for 12 days
Mean 0 19 Range 0 16-0 26 S.D ±0 035	0 21 0 15-0.27 ±0 038	

Table III, are compared, it will be seen that the variation is less, and that dimercaprol had no significant effect

DISCUSSION

Dimercaprol did not diminish the toxicity and more animals died in the experimental series than in the control Germuth and Eagle (1948) have reported that the lead-dimercaprol complex is almost as toxic as lead itself

Dimercaprol given during the exposure to lead caused less lead to be accumulated in the body, but how this was brought about, whether by an increase in excretion or by a decrease in absorption or by both cannot be determined from the evidence presented here. There is some published evidence that dimercaprol produces a temporary increase in urinary excretion of lead both in animals (Germuth and Eagle, 1948, Ginsberg and Weatherall, 1948) and in man (Ryder, Cholak, and Kehoe, 1947, Telfer, 1947)

The fact that dimercaprol had no effect on the loss of lead from the animals previously treated with lead for eight to ten days provides no support for its use in the treatment of chronic lead poisoning

SUMMARY

- 1 Lead acetate marked with a tracer of Pb²¹⁰ (radium D) was fed with a low calcium diet to mice, and after varying periods the lead content of the whole animal was determined by chemical analysis and count of β radiation
- 2 The lead content of mice which had received 50 mg dimercaprol per kg daily, and lead simultaneously for 10 to 14 days, averaged 0.48 mg

Pb/100 g of mouse, and was significantly lower than that of controls receiving lead alone, which averaged 10 mg Pb/100 g

- 3 When lead alone was administered for eight to ten days, subsequent treatment with dimercaprol during a recovery period of one to two weeks had no significant effect on the final lead content
- 4 These results provide no indications for the use of dimercaprol in treatment of chronic lead poisoning

I am indebted to Dr Rowlands and to present members of the physics department of St Mary's Hospital Medical School for advice and assistance with the estimation of the radio-activity. I am also indebted to Sir Jack Drummond and Messrs Boots for a supply of dimercaprol

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OF DIMERCAPROL AND PARATHYROID **EFFECTS** SUBACUTE DISTRIBUTION OF LEAD EXTRACT ON THE (ACETATE) IN RABBITS

BY

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It has been shown that dimercaprol (2 3-dimercaptopropanol, BAL) increases the excretion and alters the disfribution of lead in rabbits when the dimercaprol is given less than twenty-four hours after intravenous administration of lead acetate (Ginsburg and Weatherall, 1948) This paper extends these observations to a later phase in the distribution of a single intravenous dose of lead At this phase most of the lead remaining in the body was found in the bones. It seemed likely that dimercaprol would not have much effect on lead so deposited, and, at the suggestion of Professor J H Gaddum, the effect of parathyroid extract, which is believed to mobilize lead from bones (Hunter and Aub, 1927), was also examined, alone and in conjunction with dimercaprol

METHODS

The procedure in these experiments followed that of Ginsburg and Weatherall (1948) with some modifications, as follows Pb-10 (radium D) was used as a tracer instead of Pb⁻¹² (thorium B), the short halflife of which made it useless for the present purpose Radium E and radium F were removed from an equilibrium mixture of radium D, E, and F by displacement with nickel, added as foil to a solution in hydrochloric acid A small quantity of the resulting solution of radium D and some dextrose were added to a solution of lead acetate so that the final solution contained 207 mg Pb and 2-3 microcuries of radium D per ml in 4 per cent (w/v) dextrose Doses of 10 ml/kg (i.e. 0.01 mM/kg) were administered to rabbits of both sexes and various breeds by injection into the marginal vein of one ear at a rate of 20 ml per min After injection the rabbits were placed in metabolism cages and allowed food and water ad libitum from vessels placed outside windows in the cages so that spilt food or water did not dilute

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the urine nor contaminate the faeces urine were collected at first every second or third day, and later, during the period of treatment, daily The animals were killed by a blow on the occiput 13 or 21 days after the injection of lead acetate Solutions of dimercaprol for injection were freshly prepared in 66 per cent (v/v) aqueous propylene glycol dimercaprol and parathyroid extract ("Parathormone" Lilly) were injected into the paravertebral muscles in doses as indicated below

Ginsburg and Weatherall's (1948) procedure for taking and ashing samples of tissue was followed without modification Samples of liver, epiphysis and diaphysis were routinely, and of bone marrow and kidney were sometimes, made in duplicate Estimates of the concentration in other tissues were based on single samples Before estimating their radioactivity, samples were allowed to stand for at least five weeks to allow an equilibrium mixture of radium D and E to form because the counter used was not sufficiently sensitive to detect the low energy β -rays, emitted by the radium D but counted the more energetic emission from radium E The samples were diluted to a known volume usually 10, 25, or 50 ml, and their activity was measured by means of an MRC type 1 fluid Geiger-Müller counter The total count per minute, corrected for the background count, with the counter used was linearly related to the amount of radioactive material present and was negligibly affected by variations in the density of the solution within the range involved in these experiments. The concentration and quantity of lead in tissues was calculated as before except that the amount of lead in bone was assessed from the mean of the values in all the types of bone sampled for reasons discussed below

The standard error of the lead estimations calculated from duplicate determinations made during the experiments was ± 12.3 per cent. The total amount of lead accounted for in the entire animal and its excreta averaged 78 per cent of the dose, with a standard deviation of ± 12 per cent. The discrepancy between duplicate determinations is larger than in the earlier work mainly if not entirely because much

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smaller quantities of radioactive tracer were used and the time of counting samples was not increased sufficiently to attain the same accuracy. The average fraction of the dose accounted for (78 instead of 90 per cent) is rather lower. The calculation of the total amount of lead in bone involves a particularly crude approximation, and in these experiments such a faulty approximation had a far larger effect than in the acute experiments where only 5 instead of 25 per cent of the dose was involved. The poorer recovery is clearly less satisfactory, but in the circumstances it does not appear to be so poor as grossly to invalidate the results obtained.

RESULTS

Results are presented for the distribution and excretion of lead in nine rabbits, all of which received, by intravenous injection, a single dose of lead acetate containing 2 07 mg of lead per kg of body weight. Some of the rabbits were treated with dimercapiol or with parathyroid extract or with both, as indicated in Table III, and all were killed after thirteen or twenty-one days. Rabbits receiving no dimercapiol received instead similar injections of propylene glycol (66 per cent v/v in

water) The parathyroid extract was given in the morning The dimercaprol was given at the same time, though injected at a different site, and again four hours later, in order to cover the period in which the greatest mobilization of lead might be expected. The experiments were conducted in three groups, one of which consisted of rabbits 377 and 379, one of 378, 380, and 384, and one of 396, 397, 398, and 399. All the rabbits within a group were treated at the same time, and comparisons between rabbits in the same group are therefore less subject to incidental sources of variations than are comparisons between rabbits in different groups

The excretion of lead before treatment was started is shown in Table I It has been shown (Ginsburg and Weatherall, 1948) that, within twenty-four hours of giving lead, excretion occurs chiefly in the urine. In the present experiments, over a longer period, faecal excretion was usually two to three times greater. The amounts excreted appeared to depend to some extent on the quantity of excreta passed.

TABLE I

THE EXCRETION OF LEAD BY RABBITS AFTER THE INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2 07 MG pb/kg), before any further treatment

Excreta were collected at 10 a m The period "Days 0-3" therefore ends at 10 a m on the third day after injection and the period "Days 3-5" runs from 10 a m on the third day to 10 a.m on the fifth day The excreta of rabbits 378,380, and 384 were collected and estimated in bulk during the first fourteen days and so are not included in this table

			Tota	l quantity	of exc	reta and c	oncenti	ration of l	ead in	excreta		
Rabbit No	3'	79*	3	377	:	URIN 398		396],	397] :	399 -
Days 0-3 ,, 3-5 ,, 5-7 ,, 7-9 ,9-11 ,, 11-13 ,, 13-14	ml 162 178 42 36 112 102	μg /ml 0 44 0 16 0 37 0 42 0 15 0 22	ml 194 286 56 —	μg /ml 0 50 0 19 0 17 — — —	ml 220 97 198 60 184 73	μg /ml 0 99 0 82 0 29 0 23 0 40 0 17	ml 280 192 182 146 180 52	μg /ml 1 27 0 57 0 38 0 38 0 23 0 20	ml 131 38 126 99 24 56	μg /ml 2 36 1 41 0 71 0 37 0 69 0 31	ml 165 67 246 90 137 45	μg /ml 0 97 0 63 0 33 0 30 0 42 0 36
Days 0-3 ,, 3-5 5-7 7-9 ,, 9-11 ,, 11-13 , 13-14	g 125 26 61 71 124 40	μg /g 2 58 3 63 2 93 0 31 0 34 0 17	g 35 18 30 —	μg /g 3 37 4 97 3 51 — — }	g 43 119 37 145 155 68	FAI μg /g 11 02 12 56 1 61 1 16 0 58 0 13	83 103 142 100 45	μg /g 2 34 2 60 1 52 0 56 0 40 0 63	g 60 31 42 155 181 42	μg /g 7 21 3 45 6 24 2 23 0 98 0 89	g 65 86 97 125 89 50	μg /g 1 70 1 84 0 80 0 16 0 63 0 49
Urine Faeces		<u>-</u>		Total p	1	ige of the 10 8 36 1		creted in 16 9 17 1	!	12 3 32 7		9 2 8 0

^{*} Treated with propylene glycol on days 8-11

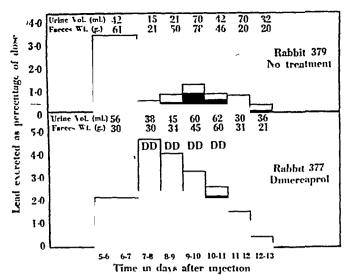


Fig 1—The effect of dimercaprol on the excretion of lead in the urine and faeces 8–11 days after the intravenous administration of lead acetate (2 07 mg Pb/kg) Ordinates Amount of lead excreted as a percentage of the dose administered (1 per cent = 20 7 μg Pb/kg body weight) Abscissae Number of days after the injection of lead acetate D = 12 5 mg/kg dimercaprol injected intramuscularly Urinary excretion

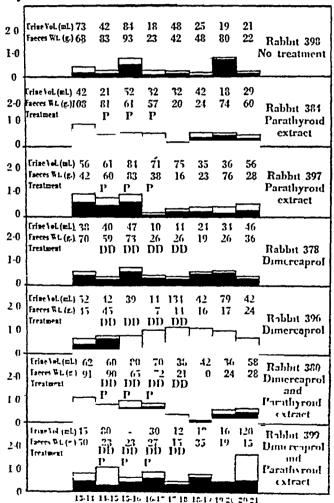
The effects of the treatments on the elimination of lead in the urine and faeces are shown in Figs 1 and 2. When dimercaprol was administered seven days after the lead (377), a tenfold increase in the urinary excretion of lead occurred during the first day of treatment, after which the amount fell progressively during subsequent days of treatment. The faecal lead also increased, although probably not significantly. In the rabbits treated with dimercaprol on the fifteenth and subsequent days

TABLE II

THE CONCENTRATION OF LEAD IN THE BLOOD CELLS OF RABBITS POISONED WITH LEAD ACETATE DURING TREATMENT WITH DIMERCAPROL AND PARATHYROID EXTRACT

	μg Lead per g of blood cells				
Rabbit No Treatment (details as in Table III)	378 Dimer- caprol	384 Para- thyroid extract	380 Dimercaprol and parathyroid extract		
Immediately before treatment 3 hours after first	0 16	0 50	0 18		
dose	<0.05	0.26	0 07		
24 hours after first dose 3 days after end of	0 09	0 20	<0 07		
treatment (i.e., at death)	0 19	0.21	0 07		

after the administration of lead (378 and 396) there was an increase in the concentration of lead in the urine and faeces This was accompanied by a diminution in the quantity of excreta, so that there was no increase in the total amount of lead A slight fall of doubtful significance occurred in the total amount of lead excreted by the rabbits treated with parathyroid extract or with dimercaprol and parathyroid extract, probably owing to the diminution in the quantity of excreta which also occurred in these rabbits Rabbit 399 developed acute retention of urine during the days preceding death, when over 400 ml of urine was found in the bladder. No cause for this retention was apparent, but it accounts for the large amount of lead recorded as excreted on the twenty-first day



Time in days after injection

FIG 2—The effect of dimercaprol and parathyroid extract on the excretion of lead in the urine and faeces 15-18 days after the intravenous administration of lead acetate (2.07 mg Pb.); Ordinates, abscissae, and symbols as in Fig 1 P = 8 unite fig parathyroid extract injected intramuscularly

TABLE III
MERCAPROL AND PARATHYROID EXTRACT ON THE CONCENTRATION OF LEAD

THE EFFECT OF TREATMENT WITH DIMERCAPROL AND PARATHYROID EXTRACT ON THE CONCENTRATION OF LEAD IN THE TISSUES OF RABBITS THIRTEEN AND TWENTY-ONE DAYS AFTER THE INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2 07 MG PB/KG)

	Microgrammes of lead per gramme fresh weight of tissue								
Time after giving lead	13	days	21 days						
Treatment with parathyroid extract	None	None	None	N	one		/day on 16, and 17	8 u /kg /day on days 15, 16, and 17	
Treatment with dimercaprol	None	12 5 mg / kg twice daily, days 8-11	None		/kg twice ays 15–18	N	lone		/kg twice ays 15–18
Rabbit No Init wt., kg Final wt, kg	379° 1 40 —	377& 1 50 —	398\$ 2 02 1 90	3789 1 90 1 60	3968 1 82 1 65	384& 1 70 1 80	3979 2 06 1 80	3809 1 90 1 80	399a 2 02 —
Plasma Blood cells Spleen Bone marrow Liver Bile Pancreas Colon Colon contents Kidneys Adrenals Gonads Seminal vesicles. Uterus Lungs Skeletal muscle Diaphysis Epiphysis Ribs Vertebrae Skull vault Brain Skin	0 06 2 25 9 8 3 0 <1 54 <0 17 0 07 0 34 0 63 <1 45 <0 95 <0 16 0 42 0 02 9 0 9 6 7 6 11 9 9 8 <0 09 0 12	0 02 0 72 3 44 6 0 4 1 0 87 0 19 0 14 0 29 1 68 0 48 0 50 	<pre><0 16 0 62 <0 64 3 50 1 9 <0 64 - <0 07 <0 30 0 67 <0 13 <0 09 5 1 12 9 19 0 12 8 <0 13 0 20</pre>	<pre><0 09 0 19 0 30 <0 25 0 62 1 0 <0 08 0 01 0 03 0 16 <1 14 <0 64</pre>	0 22 0 34 1 30 7 0 2 0 2 9 0 10 0 12 0 45 	<pre><0 10 0 21 <0 90 4 6 3 3 0 33 <0 45 0 03 0 04 0 53 <2 07 <0 64 <0 11 0 02 <0 02 7 0 10 9 10 0 7 8 0 10 0 10</pre>	<pre></pre>	<pre><0 05 0 07 <0.23 1 10 4 7 0 59 <0 15 0 01 0 27 <0 87 <0 55 <0 05 <0 002 <0 01 4 4 3 5 6 4 5 4 12 5 <0 02 0 10</pre>	0 16 0 35 <1 50 2 70 1 4 <2.21 0 50

The effects of the treatments on the concentration of lead in the blood cells are shown (for three rabbits) in Table II After the first treatment with dimercaprol, or parathyroid extract, or both, the concentrations of lead in the blood cells fell They remained low by one-half to two-thirds during treatment and afterwards, if anything, The counts were very low and tended to rise were prolonged to give an accuracy of only ±25 per cent, so that even the initial fall is barely sig-The corresponding samples of plasma contained no lead, or too little to be detected-1 e, less than 0 3-0 4 microgramme per gramme in the samples taken before death and less than 0 1 microgramme per gramme in the samples taken at death

The concentration of lead in microgrammes per gramme of wet weight of tissue and the percentage of the dose found at death (1 e, three days after the end of treatment) in various organs and calculated for various tissues are shown in Tables III, IV, and V In the previous paper in this series, Ginsburg and Weatherall (1948) calculated the amount of lead in bone from the concentration in diaphyses, because the concentration of lead in the bone marrow was much higher and it was uncertain to what extent the marrow present in the interstices of the cancellous bone of the samples of epiphyses was raising the concentration there In the present experiments the concentration of lead in the epiphyses was much higher than the concentration in either marrow or diaphyses and

TABLE IV

THE EFFECT OF TREATMENT WITH DIMERCAPROL AND PARATHYROID EXTRACT ON THE DISTRIBUTION OF LEAD IN RABBITS THIRTEEN AND TWENTY-ONE DAYS AFTER THE INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2 07 MG PB/KG)

	Percentage of dose in entire organ or tissue								
Time after giving lead	_ 13 1	Days	21 Days						
Treatment with parathyroid extract	None	None	None	No	one		/day on 16, and 17	8 u /kg /day on days 15, 16, and 17	
Treatment with dimercaprol	None	12 5 mg / kg twice daily, days 8-11	None		/kg twice lys 15–18	N	one	12 5 mg daily, da	/kg twice ys 15–18
Rabbit No Init wt, kg Final wt, kg	379º 1 40 —	3778 1 50	398å 2 02 1 90	3789 1 90 1 60	396a 1 82 1 65	384s 1 70 1 80	3979 2 06 1 80	380° 1 90 1 80	399 <i>ō</i> 2 02 —
Plasma Blood cells Spleen Bone marrow Liver Pancreas Colon Colon contents Kidneys Adrenals Gonads Lungs Skeletal muscle Bone Brain Skin Injected ear	0 12 3 11 9 6 3 8 0 01 0 03 0 18 0 24 0 02 0 01 0 08 0 50 26 0 0 70 3 86	0 06 0 62 0 12 5 8 6 0 0 03 0 07 0 12 0 55 <0 01 0 06 0 03 1 25 29 2 0 04 0 52 15 2	<pre> <0 30 0 86 <0 02 3 3 2 2 <0 02 <0 04 0 18 </pre>		<pre><0 45 0 45 0 02 6 8 2 0</pre>	<pre><0 22 0 24 <0 02 4 4 4 5 <0 01 0 01 0 01 0 16 <0 06 <0 02 0 01 <0 58 27 4 0 02 0 60 0 41</pre>	<0 30 <0 30 0 02 4 0 2 8 	<pre><0 11 0 08 <0 01 0 99 5 4 <0 01 0 04 0 07 <0 01 <0 01 <0 01 <0 28 18 7 <0 01 0 57 0 59</pre>	<pre><0 31 0 48 <0 02 2 5 1 3</pre>
Total in carcass†	50%	63%	44%	21%	29%	39%	26%	26%	45%
Excreted Urine, days 0-14 Urine, days 14-21 Faeces, days 0-14 Faeces, days 14-21	5 9* 23 0*	(10 7)*	10 8 1 1 36 1 5 7	11 3 0 8 33 9 2 7	16 9 3 2 17 1 3 6	11 8 0 8 28 9 2 7	12 3 1 7 32 7 2 0	14 9 1 3 27 1 3 1	9.2 3 1 8 0 4 9
Total excreted	29%	30%	54%	49%	41%	44%	49%	45%	25%
Total accounted†	79%	93%	98%	70%	70%	83%	75%	71%	70%

* Days 0-13 † Including items shown in Table V

clearly a larger part of the entire dose had been taken up at the ends of the bones The total amount of lead in bone has therefore been estimated from the mean concentration in all the types of bone consistently sampled, without weighting for the relative proportions of the different Large maccuracies are probably so introduced, and the figures are useful only as a rough check that a reasonable fraction of the entire dose has been accounted for

In the first pair of rabbits (377 and 379), the concentration of lead in the wall of different parts of the alimentary canal above the rectum was fairly uniform, and therefore in subsequent experiments only one portion of the intestine, the colon, was sampled The rest of the alimentary canal was the largest amount of tissue not examined in the later animals, but probably did not include more than 1 or 2 per cent of the dose

TABLE V

THE CONCENTRATION OF LEAD AND THE PERCENTAGE OF THE DOSE OF LEAD IN CERTAIN ADDITIONAL TISSUES OF RABBITS THIRTEEN DAYS AFTER THE INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2 07 MG PB/KG) WITHOUT AND WITH TREATMENT WITH DIMERCAPROL

	μg Lead fresh we tiss	eight of	Percentage of dose in entire organ or tissue			
Rabbit No Treatment	379 Propylene glycol	377 Dimer- caprol	379 Propylene glycol	377 Dimer- caprol		
Stomach Stomach con-	0 07	0 18	0 06	0 10		
tents Small intestine Small intestine	0 07 <0 07	0 07 0 14	0 11 <0 10	0 16 0 10		
contents Caecum Caecum con-	0 14 0 11	0 02 0 22	0 04 0 13	0 02 0 53		
tents Rectum Rectum con-	0 27 0 04	0 34 0 15	0 63 0 01	2.25 0 04		
tents Heart Bladder Spinal cord Eyes.	0 66 <0 18 1 18 0 38	0 32 <0 15 <0 43 0 67	0 44 <0 02 0 05 0 05	0 11 <0 02 <0 02 0 09		
	<0 12	<0 15	<0 03	<0 03		

Of the lead which remained in the body, regardless of treatment and apart from that which had not been injected cleanly and remained at the site of injection (notably in rabbits 377 and 399), 50 to 95 per cent was found in the bones No other single tissue accounted for more than a few per cent of the dose, and only the liver and bone marrow contained more than 2 per cent consistently Similarly the highest concentrations were found in the bones, bone marrow, and liver, which generally contained 2-15 microgrammes of lead per gramme of tissue Other tissues rarely contained more than 1 microgramme per gramme The concentration in blood cells, lungs, kidneys, bile from the gall bladder, and skin tended to be above 01 microgramme per gramme, whereas those in skeletal muscle, the alimentary canal, and brain tended to be below this level

General inspection of the results shows no striking differences between differently treated animals. The concentrations in bone were somewhat higher in the untreated rabbits than in any others. Interpretation of this difference is confused by the great variability of the concentration in different bones, by the large deposit of lead at the site of injection in one rabbit (399), and by variation in the amount of lead excreted before treatment was started. The total amount of lead accounted for

tended to be low when the concentration of lead in the bones was low, that is, as here calculated, there was no completely corresponding increase in the amount of lead outside the skeleton in the treated animals, and the evidence that appreciable quantities of lead are removed from the bone by any of the treatments is unconvincing. Other tissues showed no consistent differences which could be related to the treatments

DISCUSSION

The distribution of lead in the untreated rabbits of this series showed no unexpected features As has generally been found in rabbits (Kisskalt and Friedmann, 1914, Lomholt, 1924, Kehoe and Thamann, 1933), the excretion of lead, even after parenteral administration, was greater in the faeces than in the urine after the first day or two, and the combined excretion amounted to about half the total dose in three weeks. The rate of excretion roughly followed an exponential curve, and had reached a very low level at the end of these Even if there was no further experiments decrease in the rate of excretion after three weeks, it would have taken a period of the order of a hundred days to excrete the rest of the lead in the body This residual lead was, as expected, mainly in the bones, and the bone marrow and liver were the only other tissues in which concentrations usually exceeded 1 microgramme per gramme Little attention has hitherto been paid to the bone marrow as distinct from bone (cf Ginsburg and Weatherall, 1948), and the persistence of lead in the marrow is clearly interesting in relation to the mechanism by which lead produces anaemia The present figures for the concentration in muscle are lower than those of Kisskalt and Friedmann (1914) and of Kehoe and Thamann (1933), even allowing for the difference in dosage but when the small quantities of lead involved are considered, the differences are not striking nor surprising In the central nervous system the discrepancies are larger and Thamann's method was unreliable (Kehoe, Thamann, and Cholak, 1935) and their figures are very variable and may be disregarded and Friedmann consistently found concentrations several hundredfold higher than those reported With quantities of the order involved, the reliability of their method is perhaps questionable In more acute poisoning, Weyrauch (1931) failed to detect any lead in the brain of rabbits sixteen hours after the intravenous injection of lead nitrate (12 mg Pb/kg), though the sensitivity of his method was not sufficient to exclude concentrations of the order quoted here and Ginsburg and

Weatherall (1948) found slightly higher figures at six and twenty-four hours after injection than the present ones. On the whole it appears unlikely that concentrations of lead much exceeding 0.1 microgramme per gramme of tissue occur in the brains of rabbits after the intravenous injection of salts of lead.

The effects of treatment were small Eight days after the injection of lead acetate, dimercaprol increased the urinary excretion so that in all about_ an extra 4 per cent of the dose was excreted Fifteen days after the injection, the effect of dimercaprol, if any, did not exceed 2 per cent of the dose Germuth and Eagle (1948) observed larger increments in the urinary excretion of lead by rabbits up to eleven days after the last dose of lead, but they had administered several doses of 200 mg lead acetate subcutaneously and their rabbits therefore had a depot from which lead was almost certainly being continuously absorbed and was in addition possibly mobilized by dimer-Even so, as here, successive doses of dimercaprol had a rapidly diminishing effect on urmary excretion, and any increase in faecal excretion was obscured by a diminished output of During treatment the amount of lead in the blood cells decreased, and three days after treatment the concentration of lead in the cells was highest in the untreated animal of each set The difference in concentrations corresponded to a difference of about 25 per cent of the dose in the shorter experiments and about 0.5 per cent in the longer ones, and so was not far from accounting for all the extra lead excreted As indicated above, the bones may also have contributed a little A decreased concentration of lead in the bones of rabbits poisoned with large amounts of lead and treated with dimercaprol has been reported by Lusky, Braun, and Laug (1948), but no quantitative details were given the present experiments the variation between identically treated rabbits was generally larger than any changes attributable to treatment, and so attempts precisely to account for the small movements of lead which may have occurred are unprofitable The data of Ryder, Cholak, and Kehoe (1947) and of Telfer (1947) suggest-that dimercaprol has rather more effect on the lead of the blood cells and urine in man than in rabbits

The changes after parathyroid extract were also minimal. There was no increase in the excretion of lead and, if anything, a reduction in the concentration of lead in the blood cells. It does not appear that, with the doses used in the rabbit, parathyroid extract has any appreciable effect on the distribution of lead once most of the lead is

deposited in the bones Consequently the rationale of using dimercaprol and parathyroid extract together was not fulfilled, and the negative results of the combination must be attributed to the lack of action of the parathyroid extract alone lack of action was a little surprising However, the literature about the action of parathyroid extracts on lead metabolism is conflicting increase in the concentration of lead in the blood of rabbits (Teisinger, Joachim, and Kodicek, 1938) and of man (Schmitt and Taeger, 1937) has been reported, but an increase in that of the latter has not been confirmed (Teisinger, 1938), and the rise in blood lead in Schmitt and Taeger's patient was accompanied by far less increased excretion of lead than in the cases observed by Hunter and Aub (1927) Almost any interpretation can be put on these findings, and we have failed to find in the literature any complete account of the influence of parathyroid extracts on the distribution of lead in experimental animals Further observations are clearly desirable to establish whether in any circumstances parathyroid extracts really effect the mobilization of lead from bones

SUMMARY

- 1 The distribution of lead in the tissues of rabbits thirteen and twenty-one days after the intravenous injection of lead acetate (207 mg Pb/kg) has been studied by use of the isotope Pb²¹⁰ (radium D) Some of the rabbits were treated with dimercaprol, or parathyroid extract, or both, for some days during the week before they were killed
- 2 Apart from a transient increase in the urinary excretion of lead after dimercaprol, none of the treatments caused any substantial change in the distribution or the excretion of lead
- 3 About 50 per cent of the dose of lead was excreted in twenty-one days, predominantly in the faeces. The bones contained about 25 per cent of the dose twenty-one days after injection. The bone marrow and the liver were the only other tissues which consistently contained more than 1 per cent.
- 4 Treatment with dimercaprol and parathyroid extract appears to have no useful effect in rabbits subacutely poisoned with lead, but confirmation of this finding in other species is desirable

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THE ANTAGONISM OF CURARIZING ACTIVITY BY PHENOLIC SUBSTANCES*

BY

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In the course of the biological assay of solutions of d-tubocurarine chloride containing p-chloro-mcresol as a bacteriostatic agent it was found that this substance had an antagonistic action on the curarizing activity This type of antagonism has been reported previously by Rothberger (1902), Mizuno (1933), and Coppée (1943), but has never been described for isolated mammalian tissues nor for pure d-tubocurarine chloride, and in view of the present-day practice of using phenolic substances as bacteriostatic agents it seemed desirable to investigate it further Considerable confusion exists as to the identity of the so-called p-chloro-mcresol, the British Pharmacopoeia (1948a) records it as 6-chloro-3-hydroxytoluene, and (BP, 1948b) quotes Wien (1939) as having established its toxicity, whereas Wien used 4-chloro-3-hydroxytoluene which is stated to be p-chloro-m-cresol in the The p-chloro-m-cresol we Merck Index (1940) have used is 6-chloro-3-hydroxytoluene, mp 63-64° (the mp of the 6-chloro-compound is variously given as 52-66° whereas that for the 4-chloro-compound is 46°)

METHODS

Rat diaphragm-phrenic nerve preparations as described by Bülbring (1946) have been used throughout these experiments. The capacity of the bath was 100 cc. A fresh preparation was used for each compound. Our Ringer-Locke, aerated with oxygen, contained 0.5 g sodium bicarbonate/litre as opposed to the usual 0.15 g/litre. Square wave impulses of maximal strength and 0.34 or 6.7 msec duration were applied to the nerve through a fluid electrode at the rate of 5 per minute and the contraction recorded isotonically.

RESULTS

From p-chloro-m-cresol we traced the antagonism through the three isomeric cresols to

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Toluene was too toxic to the preparaphenol tion to give any satisfactory result. Next the antagonism was demonstrated in the dihydroxybenzenes, being most potent in catechol Of the trihydroxybenzenes, phloroglucinol was the only one in which we found the action, pyrogallol was too powerful a reducing agent and hydroxyhydrogumone was not available Benzyl alcohol, salicylic acid, α - and β -naphthol, 2 2'-dimethoxydiphenyl ether, veratrole, and 1 3-dimethoxybenzene were inactive. In addition to antagonizing d-tubocurarine, catechol and phenol reduced the action on a rat diaphragm of the triethiodide of 1 2 3-tri (β-diethylaminoethoxy) benzene (R P 3697), of the erythmaa alkaloids, β -erythmoidine, and dihydro- β -erythroidine, and of strychnine ethobromide the action of which is not reversed by neostigmine They produced only a very slight antagonism of decamethonium iodide addition of catechol or phenol before or after tubocurarine did not affect the result

When it became apparent that the relative antagonistic activities of a series of phenolic compounds had to be determined, a test of standardized design was decided upon tially this was to find that dose of antagonist (AD66/33) which would reduce two-thirds paralysis, produced by d-tubocurarine alone, to one-third In practice, this was found by measuring the paralysis in the presence of two doses of d-tubocurarine, the doses being selected so as to produce, by themselves, about 80 and 50 per cent paralysis respectively Logarithmically spaced doses of antagonist were added to each dose of d-tubocurarine and the paralyses displayed by the mixtures were recorded Since the relationship between log-dose d-tubocurarine and percentage paralysis is known to be linear in the absence of antagonizing substances (Fig. 1), at least between 20-80 per cent paralysis (Chou, 1947, Trevan, 1948) and

since this linearity held within the experimental limits in the presence of phenols tested at three or more concentrations of d-tubocurarine, the graphical method of interpolation of results shown in Fig 2 was employed. Here, degree of paralysis is plotted against dose of d-tubocurarine for each level of antagonist. By drawing an ordinate through the point on the upper control lines showing 66.7 per cent paralysis the percentage for each dose of antagonist corresponding to this degree of control may be read off. These figures may then be plotted against log-dose antagonist as in Fig 3 and the AD66/33 determined directly

The advantages of this design of test are that it is balanced about the 50 per cent paralysis point and that, by standardizing the effect and not the dose of d-tubocurarine (cf. Blaschko et al, 1949), the variation between rat diaphragms is eliminated

Statistical examination of the paralysis values obtained with varying doses of antagonists in the presence of the two-thirds paralysing dose of d-tubocurarine has shown that for each drug these are linearly related to log dose A χ^2 test applied to the slopes of the regression lines for eight drugs gave a value of 13 47, which is not significant at the 5 per cent probability level Thus a common slope of 30 93 (per cent per tenfold dose increment) could be given to each substance and its AD66/33 computed Comparison of the values so obtained These comparisons are recorded was then valid in Table I and include activities relative to phenol, both weight for weight and on the basis of molecular weights The fiducial limits (p 95) of the relative activities are included, and show that, while

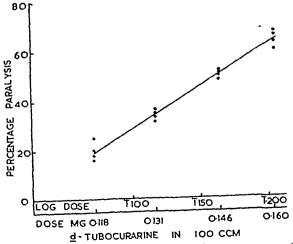


Fig 1—Dose-response-curve for d-tubocurarine on rat diaphragm showing linearity of regression Doses (in mg) are plotted logarithmically

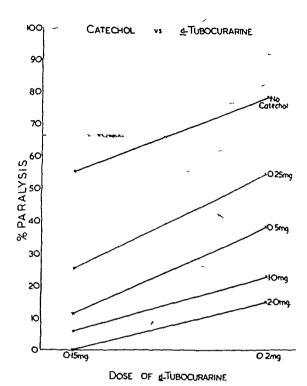


Fig 2—Reduction of paralysis of rat diaphragm by increasing doses of catechol added to two doses of d-tubocurarine. The vertical broken line is used to estimate the paralysis expected with each level of catechol in the presence of a two-thirds paralysing dose of d-tubocurarine.

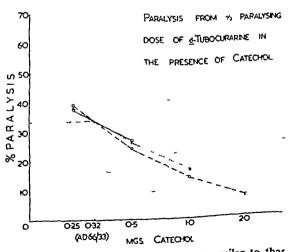


Fig 3—Data drawn from experiments similar to that illustrated in Fig 2 employing 4, 3, and 2 doses of catechol AD66/33 is the weight of catechol, read off the logarithmic scale, reducing the control two-thirds paralysis to one-third

TARLE I

PHENOLIC ANTAGONISTS OF TUBOCURARINE CHLORIDE

Activity of antagonists estimated on rat diaphragm preparations and expressed as (1) AD66/33 or that amount of antagonist which reduces by half the activity of an amount of tubocurarine which by itself would have caused 66 7 per cent paralysis, (2) activity relative to phenol on a weight-for-weight basis, and (3) relative to phenol on a molar basis In column (2) the p 95 limits are given in parentheses Hydroquinone, guaiacol, and phloroglucinol were too weak to give an AD66/33

Antagonist -	(1) AD66/33 mg	(2) Relative activity	(3) Relative molar activity
Catechol	0 30	50	5 9
p-Chloro-phenol	0 52	(3 60-7 02)	40
o-Cresol	0 62	(1 89-4 56)	28
o-Chloro-phenol	1 36	(1 37-4 46)	15
m-Cresol	1 37	(0 78–1 61)	1 3
Phenol p-Cresol Resorcinol	1 52 1 64	(0 76–1 62) 1 0 0 9 (0 57–1 52)	10
Resorcinor	2 96	(0 35-0 75)	06
Hydroquinone Guaiacol Phloroglucinol		03 01 - 01 -	0 32 0 13 0 11

the actual relationships may not be too clearly defined, there is nevertheless a distinct fall of potency down the series. The results for hydroquinone, guaiacol, and phloroglucinol were similarly obtained by extrapolation of their graphs. The values given for these three cannot be regarded as more than an indication of their relative inactivity, however, as at high concentrations their paralysis-dose curves flattened out and with hydroquinone there was a complicating contracture of the diaphragm

The results in Table I are essentially similar to those quoted by Coppée (1943) There does not appear to be any relationship between structure and action

In order to investigate the absolute relationship between antagonist and d-tubocurarine, an alternative method of treating the information obtained from these tests was employed. The reduction in paralysis produced by the addition of antagonist to a given dose of tubocurarine may be regarded as equivalent to the removal of a portion of the tubocurarine. The mean slope of the paralysis/log

dose d-tubocurarine regression line for the duration of the experiments was computed as being 252 If the paralysis produced by a known dose of d-tubocurarine be measured and its value plotted against log-dose, a line possessing the standard slope may be constructed. If, now, the paralysis be reduced by the addition of antagonist to the bath, the dose of tubocurarine which by itself would be expected to cause this smaller paralysis can be read off the graph The difference between this dose and that in fact present may be regarded as the quantity of tubocurarine "neutralized" by the dose of antagonist Each antagonist could then be represented by a curve relating its equivalence by weight with d-tubocurarine The family of curves so obtained reflected the relative potencies already observed, in that they formed a series of parallel curves, parabolic in nature, lying in order one above another on the d-tubocurarine scale The weights quoted above as AD66/33 correspond in this method to the weight of antagonist equivalent to 0.045 mg d-tubocurarine, since this is the difference between the dose of tubocurarine required to produce two-thirds paralysis and that required to produce one-third paralysis

While this method of plotting results seemed to indicate a constant activity of the different antagonists relative to one another, it was apparent that their relationship to d-tubocurarine was

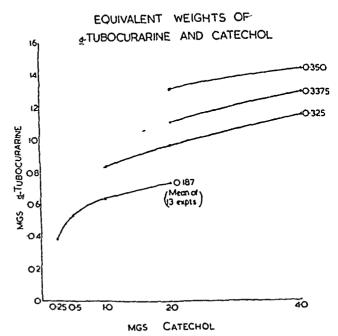


Fig 4—Neutralization of d-tubocurarine by catechol The mean doses (mg) of d-tubocurarine are indicated against the separate curves which show that the effectiveness of catechol increases as the d-tubocurarine concentration increases

not stoichiometric It appeared, moreover, that the antagonists became progressively less efficient neutralizing agents as their concentration increased Indeed, it seemed possible that the curves were asymptotic, each substance having a limiting weight of tubocurarine which it could neutralize under our experimental conditions However, closer examination of the accumulated data showed that the weight of tubocurarine neutralized by any one dose of antagonist increased as the actual dose of tubocurarine increased An experiment to illustrate this point was conducted with Fig 4 shows the mean curve for thirteen experiments in which the mean dose of d-tubocurarine was 0 187 mg, and three further lines obtained when catechol was added to increasing doses of tubocurarine. The latter three doses represented an excess of tubocurarine over the dose (approximately 0 275 mg) required to produce 100 per cent paralysis

However, when more and more excess d-tubocurarine is added a stage is eventually reached at which catechol will not antagonize the paralysis. This is reminiscent of the failure of neostigmine to antagonize large doses of d-tubocurarine (Fig. 5)

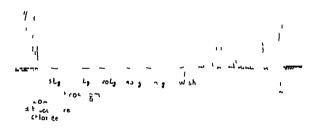


Fig 5—Rat diaphragm in 100 c c Ringer-Locke Indirect maximal stimuli 6 7 msec duration at 5 per min "Prostigmin" even at high concentrations fails to antagonize a large dose of d-tubocurarine (5 μg prostigmin normally reverses the action of 0 2 mg d-tubocurarine)

which Trevan (1948) has suggested may be due to d-tubocurarine acting at two sites, at only one of which it is reversible. Very large doses of catechol cannot be used to antagonize large excesses of d-tubocurarine because at high concentrations catechol has a depressant effect on the muscle fibres.

Further study of the nature of the action of catechol, as given below, showed its essential difference from the action of an anticholinesterase drug. It has been shown that a rat diaphragm, stimulated through the phrenic nerve with square wave impulses of short duration (0.34 msec.) and maximal intensity, responds with a twitch, whereas

longer stimuli (67 msec) produce tetanic responses (Mogey and Trevan, 1948a) Fig 6 shows a diaphragm responding with greater and lesser-contractions to long and short stimuli and also illustrates how the twitch response to short stimuli is altered to a repetitive response by eserine. This artificially induced repetitive response is abolished by minute doses of d-tubocurarine, doses so small that they do not affect the naturally occurring

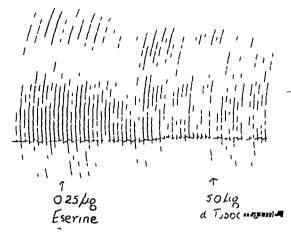


FIG 6—Rat diaphragm in 100 c c Ringer-Locke at 37° C Alternate long (67 msec) and short (034 msec) stimuli of maximal intensity at 5 per min to phrenic nerve Eserine changes twitch to repetitive response and d-tubocurarine obliterates the artificially induced repetitive response at concentrations which leave unaffected the naturally occurring brief tetanus to long stimuli

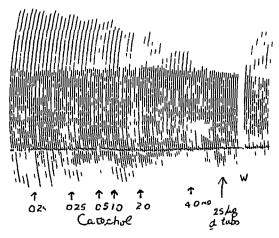
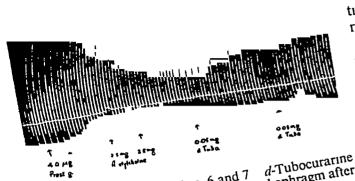
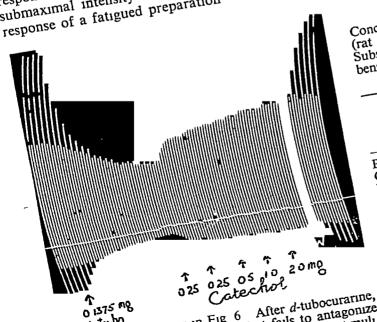


Fig 7—Conditions as in Fig 6 Catechol fails to alter twitch responses while obliterating repetitive responses d-Tubocurarine fails to restore repetitive responses, but they return after a wash



partially restores the response of the diaphragm after Fig 8 — Conditions as in Figs 6 and 7 an acetylcholine-prostigmin paralysis

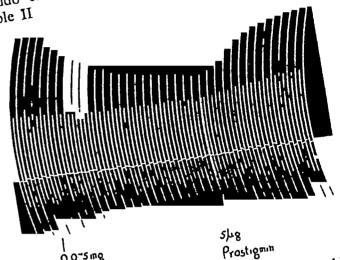
The ability repetitive responses to long stimuli to induce repetitive responses, common to many anticholinesterase agents, is not possessed by cate-Fig 7 illustrates the failure of catechol to increase the response of the diaphragm to stimuli of short duration at concentrations which increase the response during a tubocurarine-induced paralysis Instead of inducing repetitive responses, catechol abolishes them as shown in the same figure, and, catechol having removed them, d-tubocurarine in low concentrations fails to restore them is in contrast to the action of tubocurarine on an acetylcholine paralysis (Fig 8) (see also Bülbring, 1946—Fig 12) Catechol failed to increase the response of the diaphragm to short stimuli of submaximal intensity and it did not improve the response of a fatigued preparation



catechol restores the twitch but fails to antagonize Fig 9—Conditions as in Fig 6 the action on the repetitive response to long stimuli

The action of catechol on a tubocurarineinduced paralysis (Fig 9) is different from that of the anticholinesterases—e g, prostigmin as shown in Fig 10 Its action is limited to restoration of the twitch response whereas prostigmin restores the repetitive response as well

The anticholinesterase action of the hydroxybenzenes was examined manometrically by the None of those tested showed any marked inhibition of either true or method of Ammon (1933) Results are presented in pseudo cholinesterase Table II



10 —Conditions as in Fig 6 Prostigmin, unlike catechol, restores the response to long stimuli after d-tubocurarine has reduced it

TABLE II

ANTICHOLINESTERASE ACTIVITY (in vitro) Concentration of inhibitor = 10 4M Substrate for true (rat brain) cholinesterase acciyicholine of the Substrate for pseudo (horse serum) cholinesterase benzoylcholine iodide (Each result is the mean of two (rat brain) cholinesterase

Substrate for pseudo Each benzoylcholine iodide determina	result is
Substrate for pseudo (Each benzoylcholine iodide determina	percentage innivition
Compound	enzyme enzyme
Compos	1 0 4 0 0 5 0 0 0
Phenol Catechol	80 00
Resorcino	$\begin{array}{c c} 0 & 0 & 10 \\ 1 & 0 & 40 \\ \end{array}$
Phlorogram Cresol	30 \ 50 \ 50
m-Cresol	$ \begin{array}{c cccc} 70 & 70 \\ 30 & 40 \\ 30 & 00 \end{array} $
p-Chloro-menol p-Chloro-phenol o-Chloro-phenol	00 00
rine, Guaiacol	

The pH value of the Ringer-Locke, measured electrometrically, was not altered appreciably by either catechol or phenol at those concentrations which exhibited antagonism to d-tubocurarine

DISCUSSION

It is obvious that an explanation other than anticholinesterase activity must be sought for the antagonism between hydroxybenzenes and d-tubocurarine. Coppée (1943) has suggested that it is due to an "increase in the efficacy of the stimulus" by increasing the end plate potential (l'onde lente), but such an explanation fails to account for all the facts—e.g., catechol fails to restore repetitive responses to long stimuli after they have been removed by tubocurarine, it does not increase the response of the diaphragm to stimuli of short duration or of submaximal intensity (cf. adrenaline, Bülbring, 1946)

The antagonism cannot be due to an alteration of the action of d-tubocurarine by variations in H ion concentrations, for such changes are extremely slight and much too small to account for the marked antagonism. Similarly the action must reside in the hydroxybenzenes as such and not in the corresponding quinones unless it can be that the quinones, particularly o-benzo-quinone, are highly active. Unfortunately none was available for testing

We have not excluded a chemical combination between the antagonist and *d*-tubocurarine such as has been demonstrated for congo red and chlorazol fast pink by Kensler (1949) but such combination seems very improbable

One other hypothesis presented itself but was subsequently proved to be false. It was based on the fact that the ratio of the potency of the dimethyl ether of d-tubocurarine to the potency of the phenolic alkaloid varies in different species (Mogey and Trevan, 1947, Collier et al, 1948) For example, the dimethyl ether is three times as potent as the phenolic alkaloid in the rat, while it is only half as potent in the mouse suggested the possible importance of the hydroxyl groups in d-tubocurarine in some species (e.g., the rat) a methoxyl group might have a greater anchoring power than a hydroxyl, whereas in other species (e.g. the mouse) the reverse might be true Perhaps the hydroxyl group of the hydroxyvizenes was attaching itself to the same receptor so relaxing the grip of the tubocurarine If this were so then we could have expected to predict the relative potencies of anisole, guaiacol, and veratrole as antagonists of d-tubocurarine and its dimethyl ether in the rat and mouse. Such expectations were not confirmed, anisole was extremely toxic, causing contracture, veratrole was inactive against both d-tubocurarine and its dimethyl ether, and guaiacol gave results which could not be explained by this hypothesis. Cate chol and guaiacol versus d-tubocurarine and its dimethyl ether on rat and mouse diaphragms are compared in Table III

TABLE III

Catechol and gualacol as antagonists of d-tubocurarine and its dimethyl ether on rat and mouse diaphragms

A RAT DIAPHRAGM

Antagonist	d-Tuboc chlor 0 2 r	ride	d-Tubocurarine dimethylether 0 07 mg		
	Dose of antagonist	% Paralysis	Dose of antagonist	Paralysis	
Catechol	None	75	None	79	
	0 25 mg	52	0 25 mg	70	
	0 5 mg	36	0 5 mg	64	
	1 0 mg	24	1 0 mg	56	
Guaiacol	None	83	None	75	
	2 0 mg	77	2 0 mg	73	
	4 0 mg	75	4 0 mg	73	
	8 0 mg	73	8 0 mg	73	

MOUSE DIAPHRAGM

Antagonist	d-Tuboc chlor 0 375	nde	d-Tubocurarine dimethylether 0 7 mg		
	Dose of antagonist	% Paralysis	Dose of antagonist	% Paralysis	
Catechol	None	79	None	83	
	0 25 mg	26	0 25 mg	12	
	0 5 mg	8	0 5 mg	0	
Guaiacol	None	91	None	89	
	2 0 mg	66	2 0 mg	65	
	4 0 mg	54	4 0 mg	51	
	8 0 mg	50	8 0 mg	40	

The mechanism of the antagonism is so far unexplained it is possible that the solution lies in the almost universal attraction between phenols and proteins, thus causing steric hindrance

SUMMARY

- 1 A series of hydroxy-derivatives of benzene has been shown to antagonize the action of d-tubocurarine on the rat diaphragm
- 2 The relative potencies of the members of the series have been expressed as the concentration of antagonist which reduces by half the effect of a concentration of d-tubocurarine which alone t would cause two-thirds paralysis
- 3 The antagonism has been shown not to be due to cholinesterase inhibition or alteration of It is suggested that it is not due to a chemical combination of antagonist with d-tubocurarine, but the true mechanism has not been elucidated

We are indebted to and are pleased to thank Dr J W Trevan and Dr A C White for their interest and advice, and Messrs F Huggins, D J Tulett, and G Downs for technical assistance We also wish to acknowledge the kind co-operation of Dr F C Copp in supplying us with some of the compounds tested

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PERSONAL ERROR IN PENICILLIN ASSAY

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In many forms of biological testing the intrinsic errors of the methods used are so great as to render insignificant any manipulative errors on the part of the assayers Some assays, however, have such small inherent errors as to make the personal factor of considerable importance. This paper will consider the problem as met with in the course of four years' routine penicillin estimations.

METHOD OF ASSAY

The test employed was that described by Pope and Stevens (1945-6) Standard penicilin was dissolved accurately to 5 u./ml in phosphate buffer pH 60, 20 ml of this solution were pipetted into 20 ml of test broth and a range of six volumes delivered from the broth dilution into six tubes, each containing 20 ml of broth Three series of six standard tubes were set up for every thirty unknowns

A typical series of volumes would be

13	12	11	10	09	0.8
1 25	1 15	1 05	0 95	0 85	0 75
12	1`1	1.0	0.9	0.8	0.7 ml

The procedure for unknown samples was to dilute each sample in buffer to approximately 5 u/ml, further dilute this 1/11 in broth, and deliver volumes of this dilution into a set of six tubes. The range of volumes employed may of course be varied. In addition to the 10 per cent ranges illustrated above (the percentage indicates the average dilution increment between adjacent tubes) we have commonly used

20 per cent range 20 16 13 11 09 07 ml 30 per cent range 20 15 11 085 065 05 ml and, less often

90 per cent range 60 20 14 07 04 02 ml

After addition of the penicillin to the test broth the latter was inoculated from a suspension of Staph aureus (Oxford strain). Usually all the day's tests were set up with penicillin before beginning inoculation, although this order was reversed for a few months only (see below). Tests were incubated overnight at 37° C in a controlled temperature room and were then read visually by moderate indirect illumination. The end-point was taken as the mini-

mum trace of growth detectable by eye, and the volume permitting this amount of growth was estimated to 0.05 ml and recorded as "end-point volume" With practice it was possible to recognize degrees of growth corresponding to 5, 10, and 15 per cent less penicillin than that producing an end-point Thus in a range of tubes with a dilution factor of 1.2 (20 per cent range) it was possible to place the end-point to within 5 per cent (approx 0.05 ml.) should it fall between two adjacent tubes. The computation of results was completely covered by a system of tables which allowed for standard and unknown end-point volumes, and preliminary buffer dilution of the unknown

RESULTS

The sources of personal error in the technique were in the preparation of dilutions, delivery of volumes, and in the reading of the tests. In skilled hands and with the 10 per cent ranges, the standard deviation of a single test has often been of the order ±3-4 per cent. Such proficiency could only be obtained after much experience in assay, however, and even then the frame of mind of the worker was of great importance if maximum accuracy were to be achieved.

The problem which confronted this laboratory at the time of its inception in November, 1944, was one of training half a dozen assistants in the technique of penicillin assay Only one had previous laboratory experience (LB, male), the rest were girls whose ages ranged from 16 to 18, of whom all except one had matriculated and were fresh from school After only one week of general training, including a few practice assays, it became imperative that some routine tests should be undertaken, although it was realized that these could not be expected to be of great accuracy demand for assays at that time was steadily mounting, and in order to make the assayers "accuracyconscious" some simple form of "scoring" their The method adopted was to work was sought test each sample submitted for assay four to eight times, the arithmetical mean of the results of these

tests being issued as the final answer Each result was then expressed as a percentage of the mean, and these percentages plotted for the assayer responsible When the number of tests performed by a worker exceeded forty in one week, only that number taken at random was treated in this way from the week's work Fig 1 reproduces this

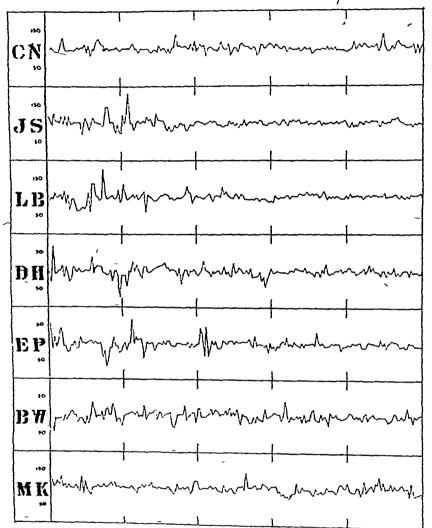


Fig 1—Worker deviation chart Results obtained by each worker plotted as percentages of the means for all workers. Forty results at random taken from each week's tests for each assayer. The horizontal scale represents weeks. Charting commenced for each worker during the first week's work.

chart over a period of five weeks, each chart was commenced during the assayer's first week. The improvement in the later weeks is very marked for some of the workers, all of whom entered into the competitive spirit which this comparison so easily fostered. However, it did not seem desirable to continue the charts after the five-week period, as the workers by then had more or less reached their maximum accuracy.

The percentages obtained for the preparation of the charts were also used to calculate the variance each week for each worker, and on the basis of the previous week's variances each assayer was assigned a weight in inverse proportion to his or her variance For simplicity these weights were reduced to simple digits The computation of the

mean for each sample was then made by appropriately weighting This apeach worker's values peared to be particularly valuable when new staff were engaged to perform assays, as only one week's practice testing was required before they could be assigned a weight, and from then on they could enter into the routine assays without fear of their inexperience unduly influencing the results issued from the Further experience department. taught us, however, that weekto-week fluctuations in individual variance were unaccountably high Consequently it was not valid to assess one week's work in terms of the previous week's variance, and the system of weighting was discontinued

A study of the mean variance for all workers each week over the period, November, 1944, to March. 1945, revealed some interesting trends (Fig 2) Initially, the mean variance was very high (SD for single tests ± 12.5 per cent) On the third week, however, this fell, and by the fourth week had reached quite a satisfactory level (SD +8 per cent) The value remained fairly steady for the next three weeks, but rose again to an alarming extent during the eighth week, only to fall during the succeeding weeks to an even lower figure than previously obtained The point of interest is that the eighth week was

in fact the three working days after Christmas We appeared, therefore, to be recording the aftereffects of festivities which, for five out of the seven staff, fell after only seven weeks' employment. Some of the workers had displayed greater excitement than the others about this time, and these were the ones whose testing demonstrated the greater variation. No such general rise in variance was observed around Christmas, 1945, by

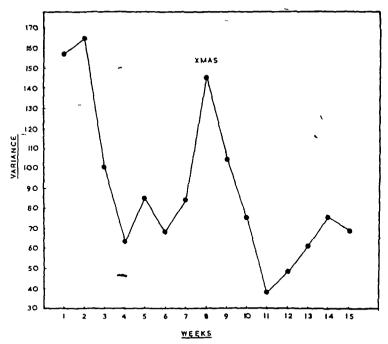


Fig 2 —Weekly variance, mean values for seven workers Each estimate plotted is associated with about 200 degrees of freedom

which time the staff had naturally "settled down" to their employment

From the eleventh week onwards some deterioration of the accuracy of our tests was noted period corresponded with the introduction of samples from the production unit, previously we had been assaying samples derived from smallscale laboratory experiments The effect of this change was twofold First, it was necessary to obtain a final answer for the production samples Second, whereas the as quickly as possible laboratory experiments were conducted with great precision, on accurately assayed material, and the samples submitted for assay could very often have their potency predicted to some extent, the penicillin content of the samples from the large-scale plant was, for some time, quite unpredictable The effect was to oblige us to test the plant samples on the wider ranges, 20, 30, and even 90 per cent steps being used The relative accuracies of these ranges will be dealt with later, but it will be clear that the overall effect at the outset was to raise the general variance

After fourteen weeks' work in the department it became too laborious to calculate individual variances each week. Instead the general variance was obtained by taking a hundred results at random from the week's work each result being expressed as a percentage of the mean value for the sample

from which it was obtained, and the variance of these percentages was calculated The value so obtained was subject to a bias, particularly when only three or four results were used to obtain the mean value for a sample Nevertheless, this statistical device seemed suitable as a general guide to the current standard of testing When new staff came into the laboratory they were allowed to spend their first week in general training with the apparatus used, and thereafter were put on to the testing of samples which had already been assayed by the more competent workers Their results on these samples were expressed aspercentages of the values already assigned and the variance of these percentages determined Usually after two to three weeks of such "duplicate" testing they were considered reliable enough to enter the routine of the laboratory weekly variances for six new arri-

vals are listed in Table I together with the general mean variance for the established staff over the first five weeks of their work. Fig 3 shows the mean variance for thirteen assayers for each of

TABLE I
WERKLY VARIANCE OF TRAINEES

Each estimate is derived from 40 results drawn from the week's work. The general mean (for the established staff) is the mean of the 5 variances for the respective weeks, each derived from 100 results.

Week of testing						General mean for
,	1st	2nd	3rd	4th	5th	weeks 1-5
RK SG NB KD SR JP	461 388 228 167 184 110	540 396 73 199 179 66	327 91 87 111 120 79	149 120 58 101 98 117	182 	103 85 104 111 115 103

their first five weeks of testing. It demonstrates very clearly that accurate testing could not be expected during the first fortnight, after which the variance drops rapidly until, at four to five weeks, the curve markedly flattens out, and, at five weeks, reaches the value of 80 for the variance, which was exactly the average value for the

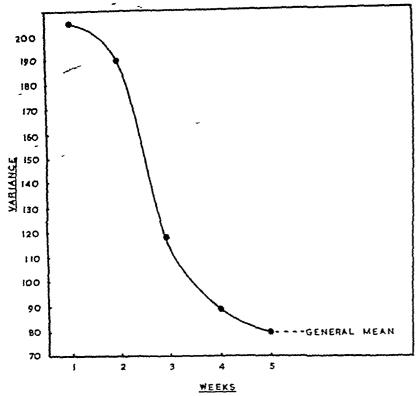


Fig 3—Weekly variance during training period, mean values for 13 workers. The general mean is that for the established staff for the 5th week. Each estimate plotted is associated with about 500 degrees of freedom.

general mean variance corresponding to the fifth week

It should be noted at this stage that the procedure in the laboratory was to record details of

samples received in a log book and to label the samples with a sample number Test sheets were then prepared showing the sample numbers, the buffer dilution required for each, and the volumes in ml to be delivered to the test One worker would then prepare the buffer dilutions of the samples and another, the "tester," would be responsible for delivering the test volumes The latter worker would also be responsible for reading her own tests after overnight incubation Thus the variance in the results would be shared between the worker preparing the dilutions and the "tester." It has always been felt that the contribution of dilution preparation to assay variance was relatively insignificant, a fact more or less borne out by consideration of the effect of the range used on the variance of results, this effect is illustrated in Fig 4, where the variance associated with different levels of titre (u /ml) is plotted In general the samples of lower titre were tested on the wider ranges and these tests were mevitably liable to greater vari-The titre range 5-100 u/ml is given the variance for dried products which were dissolved at concentrations falling within these limits These solutions were of potency tested on the 20 per cent range unexpectedly low figure for the 90 per cent range was brought about by the fact that some of the results taken for its computation were derived from closer range tests, it not being possible from the records available to say exactly which range had been used The variances of 51 and 85 for the two highest titre groups reflect an increase due to the necessity for serial buffer dilution in the preparation of samples in the higher of the two groups The increase, however, cannot be directly assessed as the result of two buffer dilutions

compared with one in the lower groups because the physical properties of the concentrated penicillin solutions make their measurements far more difficult

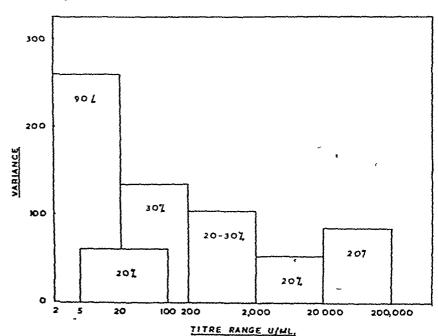


Fig 4—Variance related to titre of the sample and range employed for testing Each variance is derived from 80 observations

The general variance each week was particularly of use when, for any reason, a change in technique became necessary It was found, for example, in October, 1945, that the variance had risen to an undesirably high level, and the cause of this rise was not known By an internal reorganization of the department it was arranged that each tester should perform only half the number of tests as hitherto, since it was believed that the prevalent inaccuracy might be due to an excess of work This arrangement did, in fact, reduce the SD for a single test from ± 142 to ±118 per cent, which figure

was still considered unsatisfactory The next step was to introduce delivery pipettes in place of blow-out pipettes for the setting up of tests immediate effect of this was to raise the SD to ±137 per cent, but after a week's use of the new type of pipette the figure fell again to ±118 per cent for the second week and ±12.2 per cent for the third week It appeared that the high variance first obtained with the new pipettes was due to unfamiliarity with their calibration, but that after some practice no advantage was gained by their Finally it was decided to discontinue the practice, which we had adopted some months previously, of inoculating the test broth with a suspension of Staph aureus before introduction of the penicillin volumes We found that by inoculating - the broth after penicillin had been added the SD immediately dropped to ±90 per cent, this was considered satisfactory for the type of assays in hand and no further modifications seemed desirable It may be interesting to record that when, some four months later, considerable impending reductions in staff were announced, the effect was to raise the SD from about ±97 to ±150 per cent, but that after one week it again fell to below ±100 per cent This again reflected the emotional interference with the accuracy of the work

In view of the personal factor involved in this form of assay I was interested to find out whether the day of the week on which tests were carried out might influence the accuracy of testing. Variances were, therefore, calculated on results obtained from tests performed on each day of the week. At first a hundred results were examined for each day taken at random over a 12-week period. The variances so obtained did in fact show a tendency

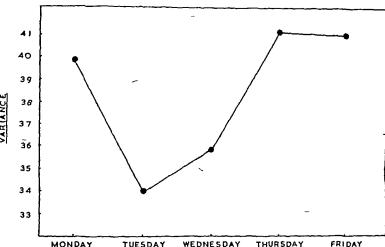


Fig 5—Daily variance, mean results for 3 girls, 280 observations per day

to reach a minimum at the middle of the week and to average at more or less the same high level both at the beginning and at the end of the week Over the period involved there were seven assayers who averaged seventy tests each day analysis was made on results eighteen months later, when over a period of ten weeks three girls averaged forty-four tests each day For each day of the week two hundred and eighty observations were made, the variances obtained are plotted in Fig 5 Again the middle of the week would seem optimal from the point of view of accuracy, although here Tuesday is even better than Since each sample was always Wednesday assayed on two successive days, the variances attributed to one day would be influenced to some extent by the preceding and following days -

Since the mid-week improvement in accuracy shown above appeared to be real it was natural to inquire whether any similar relationship held between variance and the time at which tests were carried out. It was possible to obtain assessments of the variance figure corresponding to tests set up at varying times under three different sets of con-First, during the peak period of penicillin assay, when testing took up three and a half hours in the afternoon, and approximately one and a half hours in the morning devoted to reading the tests The setting up of tests during this period was at the rate of approximately one test every two minutes Later, when owing to changed circumstances penicillin testing occupied only one hour or less of the day, it was possible to obtain variances at short time intervals (a) When the total number of tests performed on one day was not greater than thirty-two, and (b) when the total

number was greater than thirty-two, and averaged about sixty. These tests were all performed at the rate of about one per minute, and were set up on a 10 per cent range, while the first series were set up on a 20 or 30 per cent range. Fig. 6 shows the variance plotted against the time in minutes after commencement of testing. It will be seen that the variance always rose in the course of testing, and

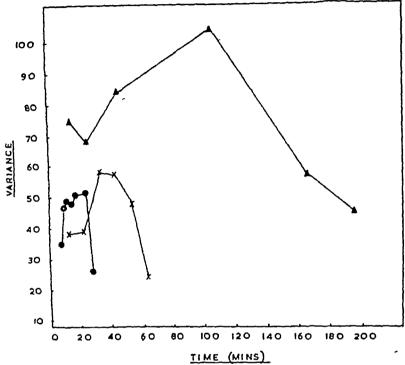


FIG 6—Variance at times during testing. The time is measured from the commencement of assays for the day. The three curves relate to different periods and represent the total penicillin assays carried out per day within each period. Each value associated with about 45 degrees of freedom.

fell again towards its completion This would imply a subconscious timing by the tester, which would give the beginning and end of her work a greater significance than the middle, and would entail greater concentration to the measurements involved Such an argument would apply equally to the setting up and to the reading of tests Since occasional check readings have usually agreed closely with the originals, it would appear that the chief errors must enter during the setting up of the tests It is probable that a form of rhythmical pipetting might commence once the testing had been started, this would particularly affect the two shorter series of assays since the volume ranges in use at that time were invariably the same for all Since the final increase in accuracy did not appear to be affected by the duration of testing, it would seem that no question of fatigue was mvolved Moreover the mean variance for thirty

tests (454) did not differ significantly from that for sixty tests (439) The mean variance for the longest series (711) was, without a doubt, higher by reason of the wider ranges used

DISCUSSION

It must be admitted that many of the effects described above are partially confounded, for

example, the error of one worker's testing may be assessed only by comparison with the results of other testers. and is, therefore, subject to error on that account Unfortunately, too, the analysis of errors between days, and within days, was only completed after the laboratory had ceased to assay penicillin samples Consequently 1t was not possible to impose any design upon the assay procedure which would have yielded results free from confounding Nevertheless the paucity of literature on the subject of personal error in routine occupations has prompted the author to present his findings

Much information has been published on tests designed to detect accident-proneness among varied groups of workers. Of this, some experiments carried out at the request of the Flying Personnel Research Committee appear to be most pertinent to the present problem. Outlined by Bartlett (1943) the work is fully reviewed by Davis (1948) in an Air Ministry publication. The purpose of the experi-

ments was to enumerate the deviations from -an ideal pattern of behaviour of pilots subjected to tests in an experimental cockpit, and to relate their findings to the subsequent accident history of individual pilots In the first instance, therefore, it was the variance of behaviour that was being These authors found that the number assessed of deviations exceeding arbitrary limits within fixed time periods rose from the beginning of the test, reached a maximum at about the middle, and fell off towards the end If the number of deviations they observed be taken to be a function of the variance, then such observations would parallel the results shown in Fig 6

Whether the psychological interpretations of their results put forward by Bartlett and Davis could be applied to the simple routine of penicillin assay will not be discussed here at length Nevertheless, it seems likely that there is a mental process common to these extremely different types of behaviour, since in each the disorganization of skill appears to be independent of physical fatigue

It has already been stated that the variance for individual workers may change considerably from week to week and from day to day The magnitude of this "variance of variance" is probably a better guide to the efficiency of the worker than the mean variance over a long period. The latter may, in fact, fail to reveal any significant differences between workers. Thus it has been observed that a worker with a mean variance lower than that of her colleagues had a variance of variance (variance measured weekly over a twelve-week period) considerably greater This type of worker has usually been observed to be subject to "moods," or periods of depression, between which their work is excellent, but during which it is most unreliable For none of the female workers has a monthly cycle been observed in individual variance, however, but sickness, possibly of nervous origin, has been associated with an increase in errors. One girl subject to migrainous headaches was quite incapable of reading her tests during attacks

SUMMARY

A method is described for the estimation of the variance of results obtained in the course of routine penicillin assays. In particular this is applied to investigate the effect of personal error on the results of the tests. Its use in the control of laboratory technique is also indicated

I would like to thank Miss Dawn Hayles and Miss Joyce Peacock for their assistance in many of the computations involved in this study, and to acknowledge the encouragement of my colleagues on the staff of the Wellcome Penicilin Unit in whose service the assays were carried out.

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THE ACTION OF NORADRENALINE

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Several investigations of the properties of noradrenaline (arterenol) have been published since the original observations of Barger and Dale As a list of these is given in the recent paper by Luduena, Ananenko, Siegmund, and Miller (1949) no attempt will be made to summarize previous work here, though the observations of others will be discussed in describing our own experi-These have been carried out in order to investigate more closely the vascular action of noradrenaline, especially its vasodilator properties, to study further the effect of denervation upon its action, and also the variation in the relative potency of adrenaline and noradrenaline in different organs We have had at our disposal synthetic *l*-adrenaline and also a sample of dl-noradrenaline kindly given to us by Dr M L Tainter

The cardiovascular system

Although it has long been known that agents like ergotoxine which reverse the pressor action of adrenaline fail to reverse that of noradrenaline (Barger and Dale, 1910), hitherto there has been no demonstration in an animal corresponding to the results of Goldenberg, Pines, Baldwin, Greene, and Roh (1948) in man These authors found that when the two substances were infused intravenously, changes occurred which enabled them to calculate that adrenaline caused a decrease of the general peripheral resistance, whereas noradrenaline caused an increase Barcroft and Konzett (1949) infused the two substances intravenously in man, and found that, whereas noradrenaline caused a rise in systolic and diastolic pressure with a slowing of the heart rate, adrenaline caused a smaller rise in the systolic pressure and a fall in the diastolic pressure with a quickening of the heart rate

Action on blood pressure—The depressor action of adrenaline described by Moore and Purinton (1900) can be most easily observed in the cat when the animal is anaesthetized with ether and the vagi

are cut Under these conditions the blood pressure is usually high. When adrenaline is infused into a vein at a slow rate, there is a fall of blood pressure. When 5 μg l-adrenaline were infused during 2 min, the fall recorded in Fig. 1a was produced. When 10 μg dl-noradrenaline were infused during 2 min, there was a slight rise of blood pressure. The effects were then repeated in (c) and (d)

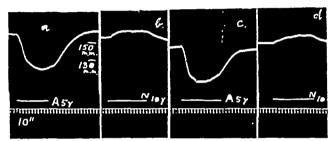


Fig 1—Cat blood pressure under ether anaesthesia after section of both vagi (a) Shows depressor action of adrenaline when 5 μ g were slowly infused during 2 min (b) Shows slight pressor action of dl-noradrenaline when 10 μ g were similarly infused (c) is a repetition of (a), and (d) is a repetition of (b)

The volume of one hindleg was also recorded in this experiment by using a plethysmograph, but except for an increase in volume pulse during adrenaline infusion no change was observed Dale and Richards (1918) showed that if the hindleg was denervated by section of the sciatic nerve seven days previously the infusion of adrenaline caused a dilatation of the hindleg By section of the nerves the reflex change in vascular tone resulting from a change in blood pressure was excluded Three cats were therefore prepared by sciatic section and the final experiment in one of these is illustrated in Fig 2 The infusion of $10 \mu g$ l-adrenaline during 30 sec produced a fall of blood pressure and dilatation of the denervated leg infusion of 20 µg dl-noradrenaline during 30 sec produced a rise of blood pressure and constriction of the leg The results, which were repeated several



Fig 2—Cat under ether, vagicut L sciatic nerve cut 8 days previously Upper record is volume of the left hindleg and lower record is blood pressure. At the first arrow 10 µg adrenaline was infused in 30 sec, and at the second arrow 20 µg dl-noradrenaline was infused in 30 sec. The first injection caused dilatation of the limb volume, the second caused constriction

times in each of the cats, show that *l*-adrenaline caused peripheral dilatation but that *dl-nor*adrenaline caused peripheral constriction, the changes in the blood pressure being due to these effects

Rabbit ear vessels—In the rabbit ear vessels it is possible to study not only the vasoconstrictor action of adrenaline but also its vasodilator action which Gowdey (1948) showed was exerted during perfusion with Locke's solution containing 2benzylimidazoline in a concentration 0.2×10^{-3} We have therefore used the rabbit ear to compare the action of noradrenaline with that of adrenaline in the presence of this reversing agent. The constrictor action of noradrenaline on the vessels of the rabbit ear has been examined by Luduena et al (1949) and also by Gaddum, Peart, and Vogt (1949) The former found that the ratio of equiactive amounts of *l-nor*adrenaline and *l-*adrenaline was 15-25, the latter found the ratio was 10-30 We made 21 comparisons and found the ratio was All these findings agree in showing that as a rule noradrenaline is a less potent constrictor than adrenaline for rabbit ear vessels, though the variation is from 25 to 100 per cent experiment in which the dog hindleg was perfused with blood by a pump, we found that 4 μ g adrenaline and 8 µg dl-noradrenaline were equally

We found that perfusion of the rabbit ear with 2-benzylimidazoline converted the vasoconstructor

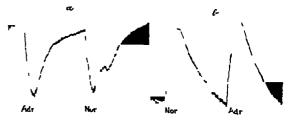


Fig 3—Rabbit ear vessels perfused with Locke's solution Outflow recorded with Stephenson's recorder (a) Shows equal constrictor effects of 0.04 μg adrenaline and of 0.25 μg dl-noradrenaline (b) Shows effects obtained during perfusion with Locke containing 0.2 × 10⁻³ benzylimidazoline

noradrenaline and 0.15 µg adrenaline

Equal dilator effects were exerted by 0.5 µg dl-

Mantingration alleich beitanberbergen bereitet

action of noradrenaline into a vasodilator action this conversion is shown in Fig 3, in which (b) shows that 0 16 μ g l-adrenaline caused a dilatation similar to that caused by 0.5 μ g dl-noradrenaline. The vasodilator ratio of l-noradrenaline to l-adrenaline was therefore about 1.5 The vasoconstrictor ratio in Fig 3a was 3.0 The results of a series of observations are given in Table I. These indicated that the relative dilator action is similar to the relative constrictor action in the majority of ears, only in occasional ears like No. 5 in Table I is the dilator action of noradrenaline very feeble. The conditions in the

TABLE I

COMPARISON OF DILATOR ACTION OF ADRENALINE WITH
THAT OF noradrenaline in rabbit ear vessels during
PERFUSION-OF 2-BENZYLIMIDAZOLINE

	 Equidila	Ratio			
Exp	l-adrenaline μg΄	dl-noradrenaline μg	/-noradrenaline/ /-adrenaline		
1 2 3 4 5 6	0 16 0 1 0 3 0 2 0 1 1 0	0 48 0 2 1 0 0 4 10 0 2 0	1 5 1 0 1 67 1 0 50 0 1 0		
	•	1			

rabbit ear appear to be the opposite of the conditions in the cat, judged by the depressor action, seen after giving ergotoxine or ergotamine. In the majority of cats the depressor action of noradrenaline is feeble or absent unless large doses are given (West, 1949), but in occasional cats it is just as great as that of adrenaline, as is shown in Fig. 4

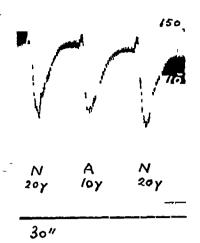
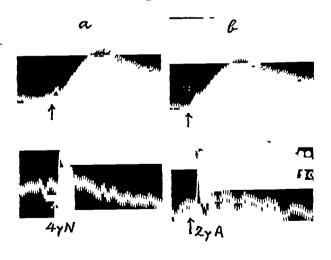


Fig 4—Cat, spinal preparation, eviscerated Blood pressure record Ergotoxine ethane sulphonate was injected in order to reverse action of adrenaline 20 μg dl-noradrenaline was injected causing a depression similar to that caused by $10 \mu g$ adrenaline This result was exceptional

Intestinal vessels—Plethysmógraph experiments were carried out on loops of the intestine of the cat under chloralose anaesthesia using the method described by Bulbring and Burn (1936) It was found that the injection of a small dose of dl-noradrenaline caused dilatation of the intestinal loop just as did the injection of a similar dose of adrenaline, as shown in Fig 5 Noradrenaline caused some rise of blood pressure, though this passed off as the dilatation came on However, to exclude the blood pressure rise as a cause of



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Fig 5—Cat, chloralose Upper record shows volume of

rig 5—Cat, chloralose Upper record shows volume of intestinal loop, lower record is blood pressure,
(a) shows dilatation of intestinal vessels when 4 μg dl-noradrenaline was injected, (b) shows similar dilatation when 2 μg adrenaline was injected

the dilatation, some experiments were done which the blood pressure was kept constant by attaching the carotid cannula to a large vessel containing Ringer's solution at constant pressure. The same dilatation of the intestinal loop was observed when noradrenaline was injected, although the blood pressure did not change.

Action on the heart—Since noradrenaline lacks the vasodilator action of adrenaline in the muscle vessels, it was of interest to discover the effect of noradrenaline on the coronary vessels. Observations were first made on the coronary flow, the rate, and the amplitude of the cat heart perfused

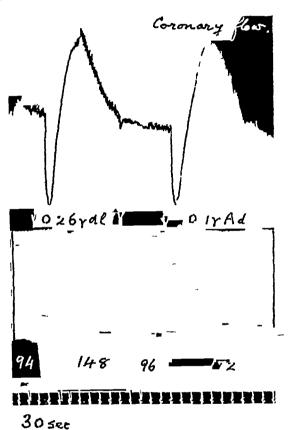


Fig 6—Cat heart, Langendorff perfusion Upper record was coronary flow by Stephenson's method, middle record was amplitude of contractions, heart rate given in figures below. The effects of injecting into the cannula 0.26 µg dl-noradrenaline and of 0.1 µg adrenaline were similar on the coronary flow and amplitude. Noradrenaline had a smaller action on the heart rate.

with Ringer-Locke by Langendorff's method Fig 6 shows the changes in coronary flow with Stephenson's recorder (1948) The effects of the two substances in the doses given were almost identical on the amplitude and coronary flow, though *dl-nor*adrenaline had less effect on the rate, increasing it by 54 beats per min, while adrenaline

increased it by 76 beats per min. When half these doses were used, the effect on the rate was the same for both, and, as there was less increase in force of contraction, the initial diminution of coronary flow was absent. Marsh, Pelletier, and Ross (1948) also observed that arterenol had the same effect on the rate, contraction, and coronary flow as *dl*-epinephrine in the perfused rabbit and cat heart. In the heart-lung preparation of the dog, observations were very kindly made for us by our colleagues Drs J M Walker and E M Lourie in the course of another investigation Fig. 7 shows the similar effect of 2 µg l-adrenaline

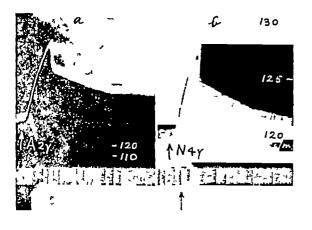


Fig 7—Heart-lung preparation of dog Upper record is the outflow from the coronary sinus collected by a Morawitz cannula and recorded with Stephenson's recorder Lower record is pressure in brachiocephalic artery In (a) the dilator effect of injecting 2 μ g adrenaline into the s vena cava is shown, and in (b) the similar effect of 4 μ g dl-noradrenaline Note that the effect of adrenaline was more prolonged (Experiment of J M Walker and E M Lourie)

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and of 4 μg dl-noradrenaline on the outflow from the coronary sinus collected by a Morawitz cannula and recorded by Stephenson's method (1949) The record shows that the effect of adrenaline was more prolonged, this was repeatedly observed Folkow Frost, and Uvnas (1949) have also found that noradrenaline exerts a dilator action on the coronary vessels of the dog

Renal vessels—Because renal ischaemia causes hypertension, we examined the effect of noradrenaline on renal blood flow to see if it caused a greater diminution than adrenaline. The action was studied in cats under chloralose anaesthesia in which the adrenal glands were excluded from the circulation and to which an injection of heparin

had been given A cannula tied into one renal vein conducted the blood through a rubber-tube back into the external jugular vein, and the rate of flow through the tube was measured by collecting the blood by a T-piece in the tube during a period of 10 or 15 sec. In one experiment the blood pressure was maintained constant by connecting the femoral artery to a reservoir of blood under an air pressure equal to that in the blood The results, shown in Table II, indicated that noradrenaline has not a greater constrictor action on the renal vessels than adrenaline, but that, if anything, the constrictor action of noradrenaline is This result is in agreement with that of Gaddum, Peart, and Vogt (1949) on the perfused renal vessels of the rabbit. They found that l-noradrenaline was equal in constrictor action to l-adrenaline when given in 25 times as great a dose

TABLE II

COMPARISON OF CONSTRICTOR ACTION OF ADRENALINE
AND HOPADRENALINE IN RENAL VESSELS

	Venous outflow c c /sec		Fall in
	Before	After	c c /sec
16 μg dl-noradrenaline 8 μg l-adrenaline 4 μg l-adrenaline 8 μg dl-noradrenaline 4 μg l-adrenaline	0 47 0 6 0 72 0 92 1 02	0 33 0 38 0 59 0 82 0 88	0 14 0 22 0 13 0 10 0 14

In another experiment the renal blood outflow was measured during the continuous intravenous infusion first of adrenaline and then of noradrenaline, in this experiment a compensating reservoir to maintain a constant blood pressure was not used. The rate of infusion was chosen so that the renal flow could be measured at the same blood pressure with the two substances. The results are given in Table III, which shows that the rates of renal flow for a given blood pressure were the same whichever substance was infused.

Summary of results.—The results on the cardiovascular system show that the one important difference between adrenaline and noradrenaline is in the action on the vessels of skeletal muscle, where the former is dilator but the latter constrictor. On the intestinal vessels of the cat on the coronary vessels of the cat and the dog, on the vessels of the rabbit ear during perfusion with 2-benzylimidazoline, noradrenaline is dilator when adrenaline is dilator. No evidence was found that

TABLE III
RENAL BLOOD FLOW

Substance	Rate of infusion µg /min	Duration of infusion min	Height of blood pressure mm	Renal flow c c /min
l-adrenaline ,, dl-noradrenaline ,, l-adrenaline	3 4 3 4 15 4 15 4 15 4 7 5 7 5	10 1 <u>1</u> 6	150 140 130 136 128 124 136 120	0 5 0 53 0 47 0 47 0 47 0 48 0 45 0 47

noradrenaline has a greater constrictor action than adrenaline on the renal vessels

The effect of denervation

The nuctitating membrane —The normally innervated nictitating membrane of the cat is much less affected by noradrenaline than by adrenaline, but after removal of the superior cervical ganglion and postganglionic degeneration the membrane becomes equally sensitive to both substances. Thus nerve degeneration brings about a small increase in sensitivity to adrenaline, but a large increase to noradrenaline (Bülbring and Burn, 1949)

The pupil dilator muscle—We have examined the changes produced by degeneration of the sympathetic fibres to the iris. About eight days after extirpation of the ganglion on one side, the cat was anaesthetized with urethane, and the pupils were examined under a bright light during injection of adrenaline and of noradrenaline. The minimum amounts of each substance which caused a dilatation of (a) the denervated and (b) the normal pupil were determined, and results in three cats are given in Table IV, which shows

TABLE IV
RELATION OF ACTION ON NORMAL AND DENERVATED PUPIL

Cat	Minimal dilating dose	Normal µg	Denervated µg	Ratio
1	l-adrenalme l-noradrenalme	30	1 I	30
2	<i>l</i> -adrenaline	2	0 1	20
	<i>l-nor</i> adrenaline	25	0 4	62
3	l-adrenaline	7 5 -	0 5	15
	l-noradrenaline	100	2 0	50

that the dose calculated in terms of *l-nor*adrenaline just sufficient to dilate the normal pupil was found to be 12–15 times greater than for *l*-adrenaline Denervation increased the sensitivity to *nor*adrenaline much more than to adrenaline, and in one cat the denervated pupil was equally sensitive to both. The behaviour of the pupil was thus similar to that of the nictitating membrane. It was always observed that injections of *nor*adrenaline, too small to cause dilatation of the normal pupil, caused constriction of the pupil

Greer, Pinkston, Baxter, and Brannon (1938) have also investigated the action of adrenaline and noradrenaline on the normal and on the denervated pupil. They wished to compare the effect of noradrenaline with that of hepatic nerve stimulation, and they drew no conclusions about the modification of the pupil response by denervation. Their photographs, however, agree with our findings

Action in other organs

The spleen—The relative activity of adrenaline and noradrenaline in causing contraction of the cat spleen perfused in situ has been stated by Gaddum, Peart, and Vogt (1949) to be 05–10, this being the ratio of the dose of *l-noradrenaline* to *l*-adrenaline. Our results in spinal cats using a spleen plethysmograph gave a wider range. Thus

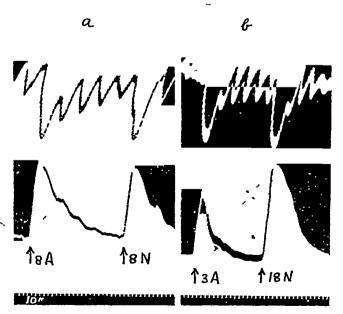


FIG 8—Cat, spinal preparation Upper record spleen volume, lower record blood pressure The figure illustrates the decline in sensitivity to noradrenaline, and the increase in sensitivity to adrenaline Early in the experiment 8 μg adrenaline was equal in action to 8 μg dl-noradrenaline Late in the experiment 3 μg adrenaline was equal to 18 μg dl-noradrenaline

in one cat *l-nor*adrenaline was found to be twice as active as *l*-adrenaline, and in another only one-fifth as active. Even in the same cat the relative potency changed in the course of the experiment, *dl-nor*adrenaline decreasing from equivalence to *l*-adrenaline to one-sixth the potency. The initial and final observations in this experiment are shown in Fig. 8. While the sensitivity to *nor*-adrenaline greatly declined, that to adrenaline slightly increased. The various results in different experiments are given in Table V.

TABLE V
CONSTRICTOR EFFECTS IN SPLEEN

Cat	Equivalent	doses in µg	Ratio	
Cat	dl-noradr	l-adren	<i>l-nor</i> adr //-adren	
1 2 3 4(a) 4(b) 4(c) 5 6 7	20 8 3 8 18 18 18 10 10	2 3 1 8 6 3 10 1 25 1 8	5 0 1 3 1 5 0 5 1 5 3 0 4 0 4 0 2 7 Range 0 5–5 0	

Bronchioles—At least two comparisons on the bronchioles have already been made Tainter, Pedden, and James (1934) found that *dl-nor-*adrenaline had one-seventh of the activity of adrenaline in perfused guinea-pig lungs in relaxing

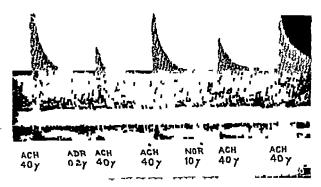


FIG 9—Bronchiolar construction recorded by method of Konzett and Roessler in guinea-pig anaesthetized with urethane Figure shows constructor action of 40 µg acetylcholine, and also the diminished action when 02 µg adrenaline was injected beforehand. The effect of this amount of adrenaline is shown to be slightly greater than the effect of 10 µg noradrenaline (Experiment of N K Dutta)

TABLE VI AMOUNTS WHICH INHIBIT BRONCHOCONSTRICTION EQUALLY

Ехр	Constrictor agent	l-adren _ µg	dl-noradren _µg	Ratio l-nor l-adr
1 2 3 4 5	Histamine ,, ,, Acetylcholine	10 0 0 5 0 2 5 0 0 2	>40 >20 >80 -120 15	>2 >20 >200 >200 12 37 5

the contraction caused by histamine or pilocarpine. Luduena, Ananenko, Siegmund, and Miller (1949) found a smaller activity—namely, that *l-nor*-adrenaline had one-seventeenth the activity of adrenaline. Our thanks are due to our colleague Dr. N. K. Dutta for making comparisons in anaesthetized guinea-pigs by the method of Konzett and Roessler (1940). The result in one experiment is shown in Fig. 9 and the different results are given in Table VI.

In these experiments there is again evidence of a very variable relationship between adrenaline and noradrenaline. In all experiments noradrenaline was weaker than adrenaline as a bronchodilator, but it was difficult to quote a single figure as expressing the mean. Some unknown factor appeared to modify the relative activity

Inhibition of cat intestine—Many workers have used the inhibitory action of noradgenaline on isolated loops of rabbit intestine as a means of estimation. The inhibition of the movements of the cat intestine in situ by noradgenaline was observed by Greer et al (1938). They attached considerable importance to it because they felt that it rendered untenable the view that hepatic sympathin or sympathin E was purely motor. We have obtained similar results to those of Greer and his colleagues, an example being given in Fig. 10, in which $5 \mu g$ dl-noradgenaline produced a slightly shorter inhibition of the duodenal contractions than $1.25 \mu g$ l-adgenaline

Effect in skeletal muscle—Adrenaline has an action in skeletal muscle which was demonstrated by Bülbring and Burn (1942). If the sciatic nerve of a cat (anaesthetized with chloralose) is stimulated by maximal single shocks at rates up to 15 per min., the tension developed by each twitch of the gastrocnemius can be recorded on the drum. When a dose of neostigmine, too small by itself to modify the tension, is first injected a following injection of adrenaline causes an increase in tension. An example of this action is shown in

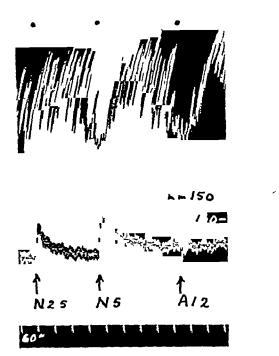


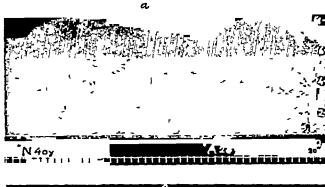
Fig 10—Cat, chloralose Upper record of volume of balloon in the duodenum Lower record of blood pressure At N 2 5, 2 5 μg dl-noradrenaline, at N 5, 5 0 μg dl-noradrenaline, at A 1 2, 1 25 μg adrenaline was injected intravenously Note that N 5 produced slightly shorter inhibition than A 1 2

Fig 11a in which an identical effect exerted by noradrenaline is also seen. When larger amounts of neostigmine are given, the injection of adrenaline may have the opposite effect, causing a decline in the tension. In these circumstances, too, noradrenaline has the same action (Fig. 11b)

DISCUSSION

Adrenaline causes vasodilatation in two conditions, first in the cat under ether when the blood pressure is high because the vagi have been cut, and second in the cat to which ergotoxine or some other reversing agent has been given. There has always been discussion whether these two conditions are essentially the same. Our observations appear to indicate that they are not, because the observations show that noradrenaline does not cause vasodilatation in the cat under ether, whereas it causes dilatation in the rabbit ear vessels in the presence of a reversing agent exactly as does adrenaline.

The vascular action of noradrenaline thus differs from that of adrenaline because it does not dilate the vessels of the denervated hindleg of the cat, but rather it constricts them. The difference is probably confined to the muscle vessels. The action of noradrenaline is similar to that of



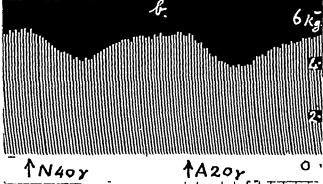


Fig 11—Cat, chloralose Record of tension in gastrochemius muscle in response to maximal single shocks applied to the sciatic nerve (a) Shows the increase of tension produced by injecting first 40 μg dl-noradrenaline, and later 20 μg adrenaline into the iliac artery. These injections were made 30 min after the injection of 20 μg neostigmine (b) Shows a record in another cat in which the same injections caused a decrease in tension. These injections were made shortly after the injection of 30 μg neostigmine. Noradrenaline and adrenaline produce the same effect on the muscle tension.

adrenaline (in similar doses) in dilating (a) the coronary vessels of the cat and the dog, (b) the intestinal vessels of the cat, and (c) the vessels of the rabbit ear in the presence of a reversing agent

Denervation, in some tissues at least, produces a greater change in the reaction to noradrenaline than to adrenaline Bulbring and Burn (1949) demonstrated that this was true for the nictitating membrane, and used the observation to estimate the relative amounts of adrenaline and noradrenaline present in a mixture. Our results show that the same change occurs in the reaction of the pupil. Only when large amounts of noradrenaline are injected does the normal pupil dilate, but the denervated pupil is about equally sensitive to noradrenaline and to adrenaline

These observations prompt the suggestion that in some tissues the presence of the sympathetic nerve supply protects the end-organ against the action of *nor*adrenaline in the blood stream, though it

does not similarly protect the end-organ against adrenaline. If noradrenaline is the chemical transmitter of the nervous impulse, such a protection might be useful from a teleological standpoint, as it would enable the nerve to have sole control of the end-organ except for the emergency when adrenaline was liberated

The great increase in sensitivity to noradrenaline after denervation may explain West's observation (1947) that pieces of intestine or uterus stored in the refrigerator for periods up to five days lose their sensitivity to adrenaline but do not lose it to noradrenaline Perhaps two processes go on side by side a general failure of the tissue metabolism measured by the loss of sensitivity to adrenaline, a failure of the mechanism of innervation leading to a denervation and therefore an increase in sensitivity to noradrenaline

If such a protection is afforded by innervation against the effect of *nor*adrenaline, it evidently plays little part in the blood vessels, the heart, the spleen, and the intestines, though this is a problem for further investigation. Certainly the spleen may become increasingly insensitive to *nor*adrenaline in the course of a few hours in spite of the opposite change taking place in the reaction to adrenaline

SUMMARY

- 1 An important difference between the vascular action of noradrenaline and that of adrenaline is that, whereas the latter causes dilatation of the vessels of the denervated hindlimb of the cat, the former causes constriction This difference is in the muscle vessels
- 2 Noradrenaline like adrenaline dilates the coronary vessels of the cat and dog and in small doses also the intestinal vessels. In the vessels of the rabbit ear, the constrictor action of noradrenaline is just as easily converted to a dilator action by 2-benzylimidazoline as is that of adrenaline
- 3 Denervation increases the action of nor-adrenaline on the nictitating membrane and on the pupil much more than it increases that of adrenaline. We suggest that some mechanism exists to protect the nictitating membrane, the pupil, and probably other organs from nor-adrenaline in the blood, this protection is less effective against adrenaline. When the nerves degenerate the protection disappears
- 4 Noradrenaline causes contraction of the spleen in situ During the course of an experiment the

spleen becomes less sensitive to noradrenaline though it increases slightly in sensitiveness to adrenaline

- 5 Noradrenaline has a smaller constrictor action on renal blood flow than adrenaline
- 6 Noradrenaline inhibits intestinal movements recorded by a balloon in the duodenum. Its inhibitory action appears to be about half that of adrenaline, though the relation-probably varies
- 7 Noradrenaline has the same effect as adrenaline on skeletal muscle previously treated with neostigmine, no matter whether the effect of adrenaline is to augment or to diminish the tension developed when shocks are applied to the sciatic nerve
- 8 Noradrenaline has much less effect than adrenaline in dilating the bronchioles, but the quantitative relation varies considerably

We wish to thank Dr N K Dutta for carrying out the experiments on the bronchioles and Mr St. John Ives for doing many of the experiments on the rabbit ear The work was done during the tenure by one of us (DEH) of a Medical Research Fellowship awarded by the National Research Council of Canada.

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THE PHARMACOLOGICAL ACTIONS OF POLYMETHYLENE BISTRIMETHYLAMMONIUM SALTS

BY

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Since the classical work of Crum Brown and Fraser, the curariform action of many onium salts has been recognized, and other activities exerted by these compounds have been described, such as those commonly called nicotine-like or muscarine-like In the present paper an account is given of the actions of members of a polymethylene bistrimethylammonium series, whose general formula is $(CH_3)_3 N^+(CH_2)_n N^+(CH_3)_3 2I$ The compounds will be referred to by the value of n, thus C10 is the decane derivative, where Our attention was drawn to the series during a test of the power of the octamethylene compound to liberate histamine After the injection of a small dose into a cat, there was no depressor response, such as histamine-liberators cause (MacIntosh and Paton, 1949), on the contrary, the blood pressure rose This rise was evidently asphyxial, since the respiration had simultaneously ceased, but there were no gasps or convulsive movements such as usually accompany asphyxia, and the sequence of events suggested some form of neuromuscular block

This series has also been independently studied by Barlow and Ing, with whom a simultaneous preliminary report was arranged (Barlow and Ing, 1948a, Paton and Zaimis, 1948a), and who have now reported their results more fully (Barlow and Ing, 1948b)

Following a further note (Paton and Zaimis, 1948b), preliminary clinical trials of the decane derivative as a substitute for d-tubocurarine chloride in anaesthesia and convulsion therapy were instituted. These have proved successful, and the name "decamethonium iodide" has been approved by the British Pharmacopoeia Commission as the official name for the compound

Our investigations of the pharmacological actions of these compounds have been chiefly directed to studying their activity in blocking neuromuscular transmission. Particular attention

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has been paid also to describing how the pharmacological activity varies with the length of the polymethylene chain Later papers will report more fully experiments on other pharmacological actions

METHODS

Effects on neuromuscular transmission

Cats anaesthetized with chloralose (80 mg/kg), after induction with ether, were used in most of the For recording the tension of muscle experiments twitch, tibialis anterior was prepared, the preparation was mounted on the Brown-Schuster myograph stand, and an isometric steel spring myograph was used for recording on the smoked drum The muscle was excited either by slightly supra-maximal shocks to the sciatic nerve through shielded silver electrodes, or directly by induction coil break shocks was tied centrally, above the point of stimulation and above the point of entry of its blood supply Injections were made either intravenously through a cannula tied into the femoral or jugular vein, or by the method of close arterial injection into the anterior tibial artery (Brown, 1938a) A few animals were anaesthetized with pentothal infused at a rate of 03-05 mg/min, or with ether from an "Oxford vaporizer" adapted for animal use, using 6-7 per cent ether

For experiments on unanaesthetized animals, injections of volumes of 001 cc/g were made into the tail vein of mice (white, male, weight 18-20 g) and rats (male, weight 100-150 g) (The effective dose was that which prevented the animal righting itself when placed on its back) Rabbits (male, weight 15-2 kg) were used for the continuous infusion headdrop method (Dutta and MacIntosh, 1949), or received rapid intravenous injections in the marginal ear vein Cats, monkeys (Macaca mulatta), and a baboon (Papio anubis) received injections in the saphenous vein Frogs were tested as described by King (1935)

Other methods

In cats anaesthetized with chloralose, the contraction of the nictitating membrane was recorded on the smoked drum. The membrane was excited to contraction by maximal stimuli applied to the peripheral

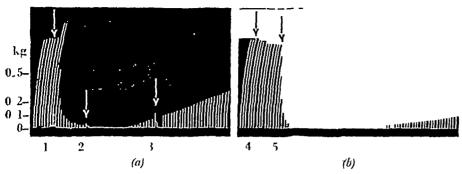


Fig 1 (a)—Cat, chloralose, 3 7 kg Record of contractions of tibialis excited by supramaximal shocks to the sciatic nerve every 10 sec At 1, 0 12 mg C10 intravenous injection At 2 and 3, tetanic stimulus to motor nerve, 50/sec (b) Same experiment, 36 min later d-Tubocurarine chloride 0.3 mg intravenously 5 min of a small dose of C10 previously At 4, 0 12 mg C10 iv At 5, 0 24 mg C10 iv

stump of the cervical sympathetic, cut and separated from the vagus in the neck, a rate of stimulation of 10 per sec produced a well-sustained contraction. In other experiments, the isolated rabbit intestine preparation described by Feldberg and Lin (1949) was employed.

Muscarine-like action was tested on rabbit or guineapig small intestine suspended in Ringer solution containing magnesium chloride (0 004 g/100 cc) atropine sulphate (10) was used as an antagonist The frog's rectus abdominis suspended in frog Ringer solution was employed in customary fashion to test for nicotine-like stimulation of skeletal muscle The spinal cat was prepared as described by Barger and Dale (1910) for the cetection of pressor activity Anticholinesterase activity was determined using a Warburg manometer, with rabbit's laked washed red cells and acetyl-\beta-methylcholine (0027 M) or rabbit plasma and benzoyl choline (0 0055 M) as sources of and substrates for "true" or "pseudo" cholinesterase respectively The substrate and inhibitor (if any) were placed in the sidearm of the Warburg bottle, so that shaking brought them into simultaneous contact with the enzyme, readings of the manometer were then made every 10 minutes for two hours The surface tension of aqueous solutions of the compounds against air was measured with a De Nöuy tension-Values for the surface tension of glassdistilled water of 71 5-72 1 dynes/cm at 20-23° C were obtained

Actions on the respiration were recorded at first by discharging the expirations of the animal (by means of light rubber valves) into a large glass vessel from which a fine adjustable leak was provided, and measuring the pressure within it by a sensitive tambour later measurements were made with the respiration recorder described by Paton (1949a) Blood pressure was measured in the usual way, a cannula coated with silicone and filled with saline containing heparin being inserted into the carotid artery. We are much indebted to Dr. J. A. B. Gray for recording action potentials from the peroneal nerve and tibialis muscle for us in certain experiments.

RESULTS

Neuromuscular block Action of C10 on the neuromuscular unction

The most notable activity of this series is that possessed by the higher members in causing neuromuscular block. Fig. 1a shows the effect of the intravenous injection of a small dose of C10 (the most active compound in this respect)

on the contractions of cat's tibialis muscle excited through its motor nerve. At first the tension is increased, and between the contractions fasciculations of the muscle can be seen (The other muscles of the animal also exhibit these incoordinated contractions.) Then the twitch tension begins to diminish until, with this dose, the muscular contraction is almost completely abolished. When the muscle is completely paralysed to stimulation through its nerve, it is still capable of responding to direct stimulation (Fig. 2)

During the progress of such a paralysis the action potential of the motor nerve to tibialis remains

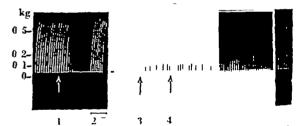


FIG 2—Cat, chloralose, 26 kg Tibialis, nerve shock every 10 sec At arrow 1, injection of 0 1 mg C10 1 v During 2, direct stimulation of muscle At 3 1 mg atropine sulphate 1 v At 4, 0 5 mg neostigmine methylsulphate 1 v

completely unimpaired while the muscle action potential and twitch tension dwindle and disappear (Fig 3A) The site of paralysis, therefore, must be placed in the end-plate region or in the terminal nerve endings

One possible mechanism for such a paralysis might be abolition of the release of acetylcholine by the nerve endings, as has been described for procaine (Harvey 1939) and suggested for atropine (Brown, 1937) We have found however, that the effects of acetylcholine given by close arterial

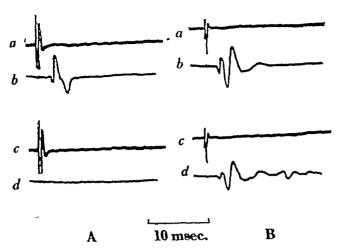


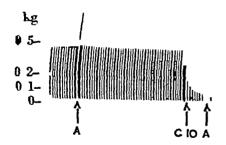
Fig 3 (A)—Cat, chloralose Supramaximal shock to sciatic nerve every 10 sec (a, c) Record of action potential of peroneal nerve (preceded by stimulus artifact), (a) before C10, (c) after 100 µg C10/kg and (b, d) record of action potential of tibialis muscle, (b) before C10, (d) after C10 Time = 10 msec -(B) Cat, chloralose, 2 6 kg Record as in (A) showing repetitive muscle action potential after 50 µg C10

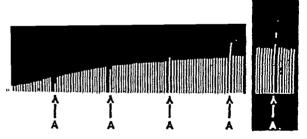
injection are antagonized as much as (or more than) the effect of a nerve volley by a dose of C10, just as they are by curare (Fig 4) A similar suppression of the response to acetylcholine is shown in Fig 9

The blocking action of C10 therefore cannot be explained by an interference with release of acetyl-

cat's plasma The plasma was then tested on the cat's blood pressure. During the control period, there was no detectable depressor activity in the effluent, stimulation of the motor nerve at a rate of 50 per sec for 2 min caused the release of depressor material in a concentration equivalent to 5 mµg acetylcholine per c c in the effluent, and the activity of this depressor material was aboushed by the injection of 0.5 mg atropine into the assay cat, this release was not prevented by adding C10 to the perfusion fluid to a concentration of 10^{-5}

The fasciculations and the potentiation of the twitch preceding neuromuscular block led us to test these compounds for anticholinesterase activity, since known anticholinesterases produce similar actions, and experiments in this connexion are described below which revealed that C10 and its neighbours possess some activity of this kind (Later work (Zaimis, 1949) indicates, however, that if this anticholinesterase action plays a part in causing these effects, such a part is small) The potentiation of the twitch may be considerable. and is best seen with smaller doses of C10, such that the subsequent neuromuscular block is too small to obscure the potentiating process, Fig 5 exemplifies such an experiment With still smaller doses, a transient enhancement of the twitch tension may be the only evidence that C10 has been injected It was necessary, therefore, to test whether the neuromuscular block might even be





Fig⁻⁴—Cat, chloralose, 2 8 kg Tibialis nerve shock every 10 sec At A, injections of 5 μ g acetylcholine intra-arterially At C10, 6 μ g C10 injected intra-arterially

choline Further, since these compounds are onium salts and do not have any local anaesthetic potency, there is no reason to expect a procaine-like action. Since the block is completely reversible, there is no reason to suspect any action such as that due to botulinus toxin. Finally, the possibility that acetylcholine liberation might be depressed was tested directly in one experiment, in which the tibialis anterior muscle of a cat was dissected and perfused in isolation with eserinized

a direct consequence of this anticholinesterase activity or not

It is well known (Briscoe, 1936, Rosenblueth, Lindsley, and Morison, 1936) that eserine and other anticholinesterases can cause neuromuscular block, which has been ascribed to the presence of an excess of acetylcholine in the region of the neuromuscular junction. Under such conditions, however, the interposition of a tetanus, or the close arterial injection of acetylcholine

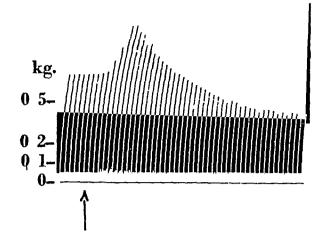


Fig 5—Cat, chloralose, 26 kg Tibialis, nerve shocks every 10 sec At arrow, 26 µg C10 injected intravenously The same dose was given 5 min previously without any effect

increases the block for the succeeding twitches (Bacq and Brown, 1937) We have used this phenomenon as a test of the nature of the block caused by C10 Figs 1a and 4 show that there is no such depressant action by a tetanus or by injection of acetylcholine respectively on the twitch of a muscle partially paralysed with C10, and it has been our constant experience that it would be hard to judge from the subsequent twitches that a tetanus had been applied or an injection made (An apparent slight deepening of the block by acetylcholine in Fig 9 was due to traces of C10 from the previous injection) We have, moreover, never observed any relation between the rate of stimulation and the development of the block __Indeed, with a large dose of C10 almost complete paralysis may occur after a single twitch unlikely, therefore, that block due to C10 is the result of the accumulation of acetylcholine at the end-plate We do not wish, however, to underestimate the resemblance of some of our tracings to those resulting from injections of potent antiesterases (cf Brown, Burns, and Feldberg, 1948), but it is possible that some of the latter produce a block otherwise than by their antiesterase action.

Our experiments in this connexion also showed that a muscle could still maintain a tetanic contraction at a height comparable with the twitch tension when partially paralysed with C10 It is well known that the curarized muscle cannot do this, our experiments on this important difference will be reported separately

Duration of action

In the anaesthetized cat, the complete or nearly complete paralysis of tibialis that follows an intravenous dose of 30 µg/kg of C10 usually begins to recover in 5-10 min, and recovery is complete Sometimes a twitch tension in about 15 min greater than the initial may be observed for a few minutes of the recovery (recapitulating the initial potentiation) before it returns to the original The presence of the drug, however, is still detectable for some time after the twitch tension has returned to normal, since the same dose given again less than 30 min after the first injection produces a greater effect But with suitable spacing of doses, reproducible cycles of paralysis and complete recovery can be obtained for many hours, the only important change commonly observed being a diminution of the initial potentiation with the lapse of time Our experience also suggests that C10 has a rather steep dose-response curve, thus, a dose of 20 μ g /kg was sometimes without visible effect on twitch tension in an animal in which 30 µg /kg produced temporarily a complete block Corresponding to this, recovery from a C10 block, once it starts, is often rather rapid d-Tubocurarine chloride differs significantly both in having a longer duration of action for a given peak action and in the slower waning of its effects

Similar time relations are observed in unanaesthetized cats and in rabbits, both animals, after a dose of C10 sufficient to paralyse them fully, recover in about 10 min, whereas d-tubocurarine chloride has a somewhat more prolonged action. In the monkey, however, the reverse is the case, and with equally effective doses C10 has a duration of action about two to three times longer than that of d-tubocurarine chloride

We have given C10 by other routes in a few experiments Administered by stomach tube, C10 is ineffective in cat and rabbit in doses less than fifty times the effective intravenous dose, but a dose of a hundred times may be lethal in an animal starved for 24 hours The paralysis takes an hour or more to appear By the subcutaneous route in the rabbit, about three times the intravenous dose is required for equal maximum effects, and the paralysis does not appear for about 10 min nor With intradisappear entirely for about 2 hours muscular injections, slightly smaller doses are required, and the onset of paralysis is quicker We have not, however, studied the relative doses required for equal peak effects by the various routes in any detail, and the values quoted are only approximate

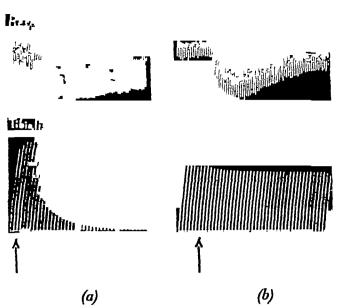


Fig 6—Cat, chloralose Record of respiration and of tibialis, nerve shocks every 10 sec (a) At arrow, 01 mg C10 intravenously (b) At arrow, 02 mg d-tubocurarine chloride intravenously

Action on the respiration

Fig 6a is the record of an experiment in which the respiratory volume was recorded simultaneously with the response of the tibialis muscle to single nerve shocks Although the muscle response was almost completely abolished, the respiratory volume was but slightly affected This has been a constant and striking experience Sometimes, indeed, an increase in the respiratory minute.

volume has been observed, a counterpart, perhaps, of the phase of potentiation of the muscle twitch which has been already mentioned With larger doses of C10, respiratory depression can of course be induced, but it is again remarkable how much sooner recovery of adequate respiration takes place than recovery of the normal muscle twitch

In Fig 6b is also shown the record of a similar experiment with d-tubocurarine chloride In contrast to C10, distinct respiratory depression was produced, with an almost negligible effect on the Our experience has been contibialis twitch sistently of this kind, that with d-tubocurarine chloride, respiratory depression accompanies or even precedes paralysis of the tibialis twitch

The contrast between the two drugs in this respect appeared so striking that it will be reported more fully elsewhere

Effect of varying the anaesthetic

Although most of our experiments were made with cats anaesthetized with chloralose, we have also used ether alone and pentothal alone the one experiment with pentothal anaesthesia, C10 appeared to be somewhat more effective than with chloralose, 20 μ g/kg being adequate for complete abolition of the tibialis twitch, although respiration was only slightly depressed with the above dose No preliminary potentiation or fasciculations were seen

With ether (6-7 per cent), on the other hand, C10 was less effective than with chloralose, and the fully paralysing dose of C10 varied from 40 to 70 μg/kg in four animals Potentiation of the twitch and fasciculations were never seen, even with only feebly paralysing doses Sparing of respiration was much less prominent than in the animal anaesthetized with chloralose were sustained very poorly, as with d-tubocurarine

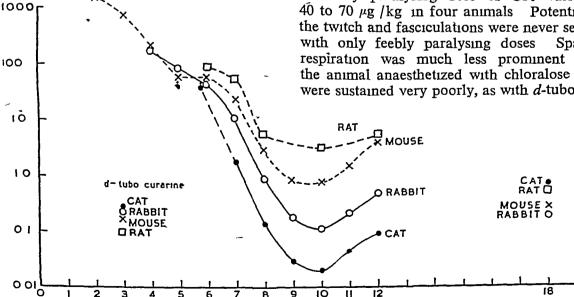


Fig 7—Variation of potency of bistrimethylammonium compounds, with varying length of polymethylene chain, and of d-tubocurarine chloride, among different species. Abscissa number of carbon atoms in chain Ordinate dose in mg/kg (Extrapolation of the curves beyond C12 to C18 has not been attempted)

	VARIOUS SPECIES
	Z
	SALTS
TABLE I	INITY OF POLYMETHYLENE BISTRIMETHYLAMMONIUM SALTS IN VARIOUS SPECIES
	POLYMETHYLENE
	OF
	ACTIVITY

	· Cat	Rabbit			Mouse	ų		!	, x	Rat	
Compound	(Dose producing 95% paralysis of tibialis) mg /kg	HDD ± standard error mg /kg	No of tests	LD 50 mg /kg	% Limits of error	ED 50 mg /kg	% Limits of error	LD 50 mg /kg	% Limits of error	ED 50 mg /kg	% Limits of error
C2 C3 C4 C5 C6 C6 C7 C10 C11 C11 C11 C12 C18 A-Tubocurarine chloride	> 40 > 40 1 9 0 16 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	>104 >40 161 approx 81 1 ± 11 9 42 4 ± 8 1 13 5 ± 2 54 0 19 ± 0 181 0 15 ± 0 0058 0 526 ± 0 0300 0 197 ± 0 0260 0 197 ± 0 0260	<u> </u>	1,490 246 246 51.2 548 238 337 0 838 0 838 0 838 0 838 0 838 0 838	(79–126) (80–125) (34–427) (36–272) (30–111) (88–114) (87–115) (75–133) (87–115) (75–133)	559 	(75-133) 	963 613 613 650 302 681 0 623	(88 6–113) (81–124) (90–110) (87–115) (76–131) (76–131) (75–133)	6 14 2 52 5 27 5 27	(90 5-111) (88-114) (81-123) (91-110)
ıodide	5			_	_	1	1	3 56	(42-237)	}	1

chloride Indeed, the effects of ether could be said to resemble rather closely those of a previous dose of d-tubocurarine chloride, which are described below

Variation of potency with species

Attempts to estimate the potency of these compounds led at once to the discovery of a very great variation with different species. For the bulk of the experiments different methods of testing were used with different species Fig 7 summarizes the results, which are shown in more detail in Table I. fogether with corresponding figures for d-tubocurarine chloride Further experiments on a, few of the compounds showed, however, that variation in the method of testing accounted for only a small part of the species difference took as a standard that dose (RD50) which, after injection rapidly by the intravenous route into unanaesthetized animals, causes loss of the ability to right themselves in half the animals. In cats this was very close to the dose required to reduce the twitch tension of tibialis by 95 per cent in the animal anaesthetized with chloralose, in rabbits, it was about 20 per cent less than the head-drop dose (HDD), and it was about 20 per cent less than the LD50 in mice and rats figures for monkey were direct estimates of RD50, those for man are based on some preliminary trials (Organe, Paton, and Zaimis, 1949) and represent the dose that made the subjects too weak to sit up or stand or lift any of their limbs If the activity of C10 in the various species is corrected to this standard, comparable estimates of its potency in these species are obtained, and are shown in Table II The results of tests on frogs are also included, but it must be remembered that the route of injection used (ventral lymph sac) was quite different from that in the other species

The actions of these drugs on various species also differed in the manner of the paralysis, and several interesting points emerged In the monkey, the earliest sign of weakness was an inability to keep the arm above the head, after this appeared progressive weakness of movement and dropping of the head, and only with deep paralysis was the ability to sit up lost In cats, the paralysis progressed. more uniformly, and neck, trunk, and limbs seemed to weaken together, but an unusual and constant feature was the complete relaxation of the nictitating membrane for so long as the paralysis lasted this relaxation is, indeed, the first sign of paralysis observed after an intravenous injection Finally, in rabbits weakness of the hindlegs appeared first while further paralysis of the limbs and head-drop

109

5 49

RELATIVE POTENCY OF C10 IN VARIOUS SPECIES, AS DOSE REQUIRED TO PARALYSE RIGHTING REACTION								
Species	Cat	Man	Baboon	Rabbit	Monkey	Mouse	Rat	Frog

0 14

0 20

0 25

0.09

TABLE II

0 14

followed The opportunity was taken of administering C10 to one baboon (Papio anubis), weight 145 kg, which was suffering from a traumatic paraplegia and was to be killed After the slow injection of C10 at a rate of 0.5 mg/min, headdrop occurred after 2 mg had been injected, followed by almost complete skeletal paralysis respiration was still adequate although depressed Injection of 50 mg of C5 9 min later caused partial recovery of arm strength and deepening of respiration

0.03

0.3

0.05

0 25

Dose mg /kg C10

d-Tubocurarine chloride

Variation of potency with length of polymethylene chain

Despite the species variation, C10 was the most potent member of the series by any test for neuromuscular block Its immediate neighbours in general closely resembled it qualitatively, although not in potency The steepness of the curve relating potency to chain-length deserves comment, for instance, shortening the polymethylene chain from eight to seven carbon atoms reduced potency more than tenfold Members of the series remote from C10, however, gave the impression that new activities were appearing The abrupt change of slope in Fig 7 in the region of C6 and the attenuation of the species difference with C18 are of great interest Since head-drop and lethality are not specific tests for neuromuscular block, it is possible that the other pharmacological actions of the series described below become prominent in bringing about an end-point with the members of the series which are relatively inactive in causing neuromuscular block

A point of interest lies in the activity of tetramethylammonium iodide relative to these com-A dose of 5 mg/kg injected intravenously into a cat produced an effect on tibialis twitch comparable with that of 30-40 μ g /kg of This potency, although small, is greater than that of C4, C5, and C6, which fail to depress neuromuscular transmission in doses of 40 mg/kg Our results with mice, rats and rabbits indicate that C2 and C3 are equally or even more mactive in this respect

Interaction of C10 with d-tubocurarine chloride

0 691

0 120

2 5

0 0766

Our first attempts to compare the potencies of these two drugs on the cat's tibialis yielded puzzling results, until it was realized that C10 was less effective than usual when d-tubocurarine chloride had been given previously Fig 1a and 1billustrates this point on cat's tibialis It can also be shown on the rabbit head-drop, and a typical experiment is cited in Table III In this experiment the preliminary dose of curare was such as caused distinct (although transient) weakness of

TABLE III

EFFECT OF PREVIOUS ADMINISTRATION OF d-TUBOCURARINE CHLORIDE (d-TC) ON SENSITIVITY TO C10

Cross-over test on four rabbits, 0 15 mg d-tubocurarine chloride per kg 1 v 15 min before test

Rabbit HD	Rabbit HDD (mg /kg)		
C10 alone	C10 after d-TC		
0 088 day 1 0 222 day 1 0 104 day 2	0 224 } day 2 0 365 } day 2 0 171 } day 1		

Dose of C10 after d-TC = 181 (limits of error for P =Dose of C10 alone 0 05 1 10-2 99)

TABLE IV

EFFECT OF PREVIOUS ADMINISTRATION OF C10 ON SENSITI-VITY TO d-TUBOCURARINE CHLORIDE Cross-over test on four rabbits, 0 075 mg C10 per kg 1 v. 15 min before test

Rabbit HD	D (mg /kg)
d-TC alone	d-TC after C10
0 264 \ day 2 \ 0 232 \} day 2 \ 0 179 \ day 1	0 207 \ day 1 \ 0 184 \ 0 131 \ day 2

Dose of d-TC after C10 = 0 753 (limits of error for P =Dose of d-TC alone 0 05 0 723-0 784)

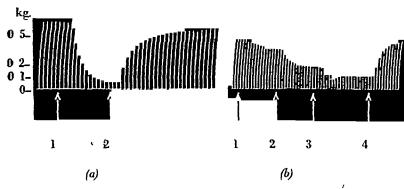


Fig. 8—(a) Cat, chloralose, 2 2 kg Tibialis nerve shocks every 10 sec At (1), 0 1 mg C10 1 v At (2), 10 mg C5 1 v (b) Cat, chloralose, 2 8 kg Tibialis nerve shocks every 10 sec At (1), (2), (3), 1 mg, 0 5 mg, 0 5 mg, respectively, d-tubocurarine chloride 1 v At (4), 0 5 mg neostigmine 1 v

the animals, nevertheless, a larger dose of C10 was required subsequently to produce head-drop. The previous administration of C10 does not (despite its feeble anticholinesterase action) lessen the effect of d-tubocurarine chloride, but rather augments it to a slight degree according to the interval between the injections (Table IV)

This antagonistic effect of *d*-tubocurarine chloride is detectable in the rabbit for an hour and in the cat may persist for a similar period. The duration of the antagonism and its effectiveness become greater as the dose is increased. In addition, the fasciculations and potentiation normally produced by C10 are uniformly abolished

These actions are not specific to d-tubocurarine chloride, we have observed them also with its methyl ether, d-bebeerine methodide, N-methyl-diaboline iodide, and with tri(diethylaminoethoxy)-benzene triethiodide ("Flaxedil")

TABLE V

EFFECT OF PREVIOUS ADMINISTRATION OF NEOSTIGMINE METHYL SULPHATE ON SENSITIVITY TO C10

Cross-over test on four rabbits, 0 05 mg neostigmine methyl sulphate per kg 1 v 1 min before test

Rabbit HDD (mg/kg)

C10 alone	C10 after neostigmine
0 117 day 1 0 156 day 1 0 203 day 2	$ \begin{array}{c} 0 \ 112 \\ 0 \ 154 \\ 0 \ 232 \\ 0 \ 112 \end{array} $ day 2

 $\frac{\text{Dose of C10 after neostigmine}}{\text{Dose of C10 alone}} = 1 00 \text{ (limits of error for } P = 0.05 0.795-1.258)$

Antagonists

Eserine and neostigmine are without effect on the neuromuscular paralysis due to C10 and its neighbours exemplifies the failure of neostigmine to reverse such block in the tibialis muscle, Fig 8b is an illustration of an effective antagonism by a similar dose to d-tubocurarine chloride (The slight deepening of block due to C10 by prostigmine in this experiment (Fig 2) was seen at other times, but not constantly) Eserine was equally ineffective Similarly with the rabbit headdrop test, a dose of 005 mg

neostigmine methyl sulphate per kg, previously given, which increased the HDD of d-tubocurarine chloride from 0316 mg/kg to 0615 mg/kg in four rabbits, did not alter the HDD dose of C10 significantly from 0149 mg/kg (Table V)

Observations on the frog's rectus, in which C10 produces a contracture, showed that lower members of the series, inactive both in causing neuro-muscular block and in producing a contracture, antagonized this action of C10 C5 and C6 were

TABLE VI

EFFECT OF PREVIOUS ADMINISTRATION OF C6 ON SENSITIVITY TO C10 AND TO d-Tubocurarine chloride

Cross over tests on four rabbits, 10 mg C6 per kg 1 v 5 min before test

Rabbit	HDD (mg	/kg)
KAUUII			,

C10 after C6
0 180 day 2 0 273 day 2 0 240 day 1

 $\frac{\text{Dose of C10 after C6}}{\text{Dose of C10 alone}} = \frac{1.69 \text{ (limits of error for P = } 0.05 \text{ } 1.23-2.41)}{0.05 \text{ } 1.23-2.41)}$

d-TC alone	d-TC after C6
$ \begin{array}{c} 0 \ 177 \\ 0 \ 336 \\ 0 \ 266 \\ 0 \ 280 \\ \end{array} $ day 2	0 163 day 1 0 392 day 1 0 181 day 2

 $\frac{\text{Dose of } d\text{-TC after C6}}{\text{Dose of } d\text{-TC alone}} = 0.878 \text{ (limits of error for P} = 0.05 \text{ 0.05} \text{ 0.0638-1.21}$

particularly effective in this respect. This suggested that such antagonism might also exist at the mammalian neuromuscular junction. Fig. 8, a record of an experiment to test the point on the cat's tibialis with C5, shows that this expectation was fulfilled. Table VI summarizes the results of a similar experiment with C6 using the rabbit head-drop method. Table VI also shows that C6 not only has no antagonistic action to d-tubocurarine chloride but may even potentiate its action somewhat

The antagonistic action of C5 (and C6) is complicated to some extent by their ganglionic action (described below), and with large doses there is no doubt that a fall of blood pressure occurs which is due to paralysis of sympathetic vascular tone This does not, however, affect the recovery of neuromuscular transmission when G5 is administered during a paralysis due to C10 A useful antidotal action can be observed in rabbit and monkey where the dose of C5 is only ten times that of the paralysing dose of C10, and under these conditions vascular effects are trivial With this ratio of doses, shallow paralyses are cut short, recovery from deeper paralyses is accelerated, and respiratory depression due to larger doses still is greatly lessened On the cat tibialis preparation, however, a larger ratio of C5 to C10 is usually required, and 3 mg/kg C5 may be required for a prompt antagonism The antagonistic action of C5 is easily reversed by increasing the dose of C10, and the renewed onset of neuromuscular block can be again antagonized by further doses of C5, there is a limit to this process, however, and with very large doses of C5 little more recovery from neuromuscular block can be obtained Similarly, after large doses of C10 it is difficult to demonstrate any antagonism (just as neostigmine is not very effective after large doses of curare)

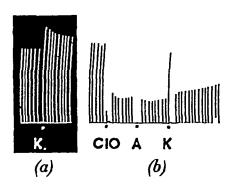
Potassium has been shown to antagonize the neuromuscular block due to curare (Wilson and Wright, 1936) We have therefore tested it against a similar block due to C10 (Fig 9b) It will be seen that there is no important action, although the dose is enough (when given to the unparalysed muscle) to produce a typical potentiation of the twitch (Fig 9a) Adrenaline appears to be equally ineffective

Paralysis of autonomic ganglia

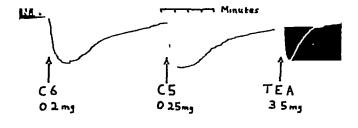
A few seconds after the injection of 10 mg of C6 into a rabbit, we observed that its ears flushed vigorously and became warm, and we have already mentioned the ability of C5 and C6 to

cause a fall of blood pressure. The analysis of these effects revealed that these compounds paralyse autonomic ganglia. The evidence for this will be presented in another paper. For the present we wish only to describe the experiments made to compare quantitatively the potencies of

FIG 9—Cat, chloralose, 28 kg Tibialis nerve shocks every 10 sec Intra-arterial injections (a) At K, 3 mg KCl (b) At C10,4 µg C10, at A, 5 µg acetylcholine, at K, 3 mg KCl



C6 and its neighbours in this respect. The cat's nictitating membrane, excited to a sustained contraction by stimulation of the preganglionic cervical sympathetic trunk at a frequency of 10 shocks per second, provided a useful test for activity of these compounds on transmission in the superior cervical ganglion. Fig. 10 shows a typical tracing in which C5 and C6 and tetraethyl-



Baseline

Fig 10—Cat, chloralose Record of sustained contraction of nictitating membrane excited by stimulation of cervical sympathetic 10 sec intravenous injections Effects of 0.2 mg C6, 0.25 mg C5, and 3.5 mg tetraethylammonium iodide (T E A)

ammonium iodide were compared Table VII gives a summary of those doses of these compounds and of tetraethylammonium iodide which caused roughly equal peak relaxations of the nictitating membrane. There was a considerable difference between C5 and C6 and tetraethylammonium iodide in their duration of action, the former two drugs acting more slowly and exerting their action three to four times as long as the latter, for doses which gave equal peak effects

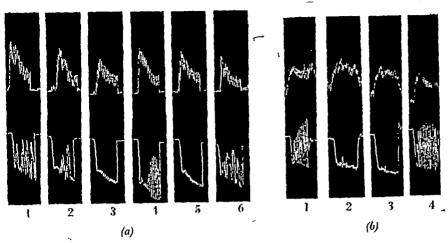


Fig 11—Isolated rabbit intestine Record of length (upper tracing) and volume (lower tracing), intestine stimulated by rapid rise of intra-intestinal pressure of 3 cm water (a) 1, normal response 2, 0 1 mg C6 added to bath 3, 0 2 mg C6 4, 15 µg, and 5, 30 µg d-tubocurarine chloride 6, normal response (b) 1, Normal response 2, 0 3 mg C6 3, 2 0 mg tetraethylammonium rodide 4, Normal response

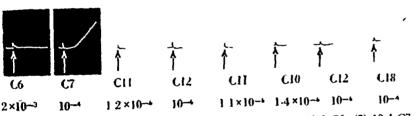


Fig. 12 —Frog's rectus Contractions elected by (1) 2×10^{-3} C6, (2) 10^{-4} C7, (3) 1.2×10^{-8} C11, (4) 10^{-6} C12, (5) 1.1×10^{-6} C11, (6) 1.4×10^{-6} C10 (7) 10^{-6} C12, (8) 10^{-4} C18

To test the effect on parasympathetic autonomic ganglia, we used the technique of Trendelenburg described by Feldberg and Lin (1949) Fig 11 shows typical tracings, and Table VII gives the relative potencies of the compounds tested It was again observed by comparing the ease with which they could be washed out that C5 and C6

TABLE VII

Relative potencies in causing relaxation of cat's nictitating membrane, excited by stimulation of cervical sympathetic trunk (Arbitary scale, $C6 \approx 100$)

Compound C4 C5 C6 C7 C8 Tetraethylam-monium iodide
Potency 2 80 100 10 2 5

Relative potencies on peristaltic reflex of small intestine (Arbitary scale, C6 = 100)

Compound C2 C3 C4 C5 C6 C7 iodide
Potency 3 0 4 3 5 9 33.3 100 16 7 14

were more persistent in their action than tetraethylammonium iodine

The intravenous injection of C6 or C5 in somewhat larger doses commonly produced a fall of blood pressure of fairly slow onset and recovery The magnitude of the fall was closely related to the initial blood pressure level

A search was made for stimulation of autonomic by these comgangha None of the pounds series, however, would produce a significant elevation of the blood pressure or change of heart rate of the spinal cat even in doses twenty times those of nicotine tartrate or tetramethylammonium iodide adequate for this, nor would similar doses elicit more than a trivial contraction of the nictitating membrane the isolated intestine, there was a tendency for the higher members to initiate peristaltic waves (particu-

larly C12), but this action was slight, and could be ascribed to their anticholinesterase activity

Stimulation of skeletal muscle

It was at first supposed that it would be possible to show on the frog's rectus an antagonism by C10 of the effects of acetylcholine similar to that exerted by d-tubocurarine chloride. It was found, however, that C10 itself stimulates the frog's rectus to contraction. Whether applied in low or high concentrations, and whether allowed to act briefly or for 10-15 min, no signs of any other than stimulant activity appeared. Indeed, so far from antagonizing acetylcholine, it usually potentiated its effects slightly, probably because of C10 s weak anticholinesterase activity.

The contraction produced by C10 and its neighbours (Fig 12) differs in several respects from that caused by acetylcholine (Fig 14), it has a slower onset, and does not so readily reach a plateau after C10 has been washed out, a longer time is

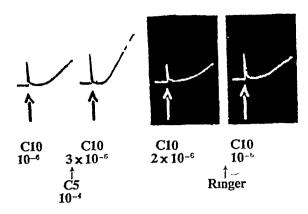


Fig 13 —Frog's rectus Antagonism of contracture due to C10 by C5 (1) 10⁻⁶ C10, (2) 3 × 10⁻⁶ C10, (3) 2 × 10⁻⁶ C10, (4) 10⁻⁶ C10 During (2) and (3) Ringer contained 10⁻⁴ C5

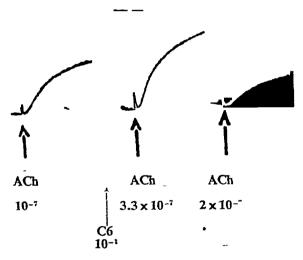


Fig 14 —Frog's rectus Antagonism of contracture due to acetylcholine-by C6 (1) 10^{-7} ACh, (2) $3~3\times10^{-7}$ ACh, (3) $2~\times~10^{-7}$ ACh During 2 and 3, Ringer contained 10^{-4} C6

needed to obtain proper muscular relaxation. Its dose-response curve is, however, not dissimilar to that of acetylcholine and it resembles acetylcholine in one important respect, that its action is also antagonized by d-tubocurarine chloride and by fairly high concentrations of atropine (10-1)

Measurements were made of the relative potency of the members of the series with respect to each other and to acetylcholine (Table VIII) During this part of the experiment, the fact mentioned above emerged, that the shorter chain compounds

TABLE VIII

Relative potencies in stimulating frog s rectus abdominis (Arbitary scale, C12 = 100)

Compound C6 C7 C8 C9 C10 C11 C12 C18 choline Potency 0 1 1 3 7 1 36 71 91 100 1 3 1,500

which are inactive in causing contractures, were nevertheless exerting an action on the muscle, since they diminished the contractions elicited by the longer chain compounds (Fig. 13). The antagonism to C10 was a maximum with C5, closely rivalled by C6. Table IX gives the concentrations

TABLE IX

Inhibitory concentrations against C10, tested on frog's rectus (Concentration causing 50 per cent inhibition)

Compound C4 C5 C6 Concentration 8×10^{-5} 5 0 × 10⁻⁵ 5 5 × 10⁻⁵

of the members tested which caused 50 per cent inhibition of C10. It was also observed that C5 and C6 can antagonize the contracture produced by acetylcholine (Fig. 14).

The stimulation of skeletal muscle could also be demonstrated in the cat (Figs 4 and 9) experiments a few μg of C10 were injected rapidly into the artery of a tibialis prepared for close arterial injection, during an intermission of stimulation, just as after a similar injection of acetylcholine there was a fast contraction of the muscle which followed the injection so rapidly as to seem synchronous with it. After the rapid contraction is complete, there is often left a small residual contraction for a few seconds, which is followed by the onset of neuromuscular block Roughly, the dose of C10 required to produce a twitch of the muscle is one which produces a substantial neuromuscular block, smaller doses are meffective in both respects, and larger doses produce a larger twitch before long-lasting block ensues not appear to be possible with any dose by this route to obtain paralysis without evidence of stimulation first, except by making the injection rather slowly

After intravenous injections of C10 we have never seen the rapid twitch just described, but large doses may elicit a small contraction lasting 10-20 sec, of a few grammes tension. No detailed comparison with other members of the series has been made, but, in general, our experiments are not inconsistent with the ranking of potency in stimulating muscle, which we obtained on frog's rectus

Anticholinesterase potency

Anticholinesterase activity was tested by two methods in order to distinguish between the activities of the compounds against the so-called "true" and "pseudo" cholinesterases For the former. acetyl-\(\beta\)-methylcholine and laked rabbit cells

TABLE X
ACTIVITY IN INHIBITING CHOLINESTERASE

Substrate concentrations for "true" enzyme 0 027 M acetyl-β-methylcholine, for "pseudo" enzyme 0 0055 M benzoylcholine

Compound	"True" concentration for 50% inhibition	"Pseudo" % inhibition at a concentration of 1.25 × 10-3
C7 C8 C9 C10 C11 C12 C18	$\begin{array}{c} 35 \times 10^{-3} \\ 10 \times 10^{-3} \\ 1.2 \times 10^{-4} \\ 45 \times 10^{-5} \\ 18 \times 10^{-5} \\ 56 \times 10^{-6} \\ 14 \times 10^{-5} \end{array}$	8 35 97
Eserine d-TC	7 1 × 10 ⁻⁸ 1 1 × 10 ⁻³	10

were used, for the latter, benzoylcholine and rabbit plasma as source of esterase The compounds had little activity against "pseudo" cholinesterase, and it was not thought worth while to study their actions at concentrations higher than 1.25×10^{-3} The values obtained are shown in Against true cholinesterase, however, Table X the longer chain members had appreciable activity, and sufficient determinations were made to be able to estimate the concentration of drug causing 50 per cent inhibition of the enzyme, this figure for each member of the series is given in Table X, and has been plotted in Fig 15 Comparable figures for eserune are included in Table X, it is about 650 times more active than C10 against true cholinesterase

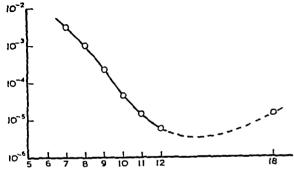


Fig 15—Variation of activity of bistrimethylammonium compounds against "true" cholinesterase, with varying length of polymethylene chain Abscissa number of carbon atoms in polymethylene chain Ordinate concentration for 50 per cent inhibition of the "true" cholinesterase "Substrate 0 027 M acetyl-3-methylcholine

A point of some interest is the selective paralysis of "true" cholinesterase, this, no doubt, is a function of the particular enzymes chosen. But the degree of selectivity is considerable, by extrapolation, it can be estimated that C10 is 200 times more active against "true" than against "pseudo" cholinesterases, and C12 about 600 times.

We have referred already to the potentiation of the twitch during the early stages of a block by C10 Records of the muscle action potential (Fig 3B) show repetitive firing of the fibre in response to single shocks, similar to that well-established for eserine and disopropyl fluorophosphonate. The possibility can be raised therefore that C10, although far less active than eserine in vitro as an anticholinesterase, becomes relatively more effective because of its lack of affinity for pseudocholinesterase in plasma. It is also possible, however, that the stimulant action of C10 on the muscle must be taken into account in explaining the fasciculations, potentiation of the twitch, and repetitive firing (cf. Zaimis, 1949)

Muscarine-like action and atropine-like action

Tetramethylammonium ion has a muscarine-like action, and it was of interest to determine whether these closely related compounds also shared it But such activity as they possess is very slight, and could only be demonstrated easily on the isolated guinea-pig or rabbit intestine, the most active compound tested (C12) was about 1,300 times less active than acetylcholine. Atropine in a concentration of 4×10^{-9} in the bath abolished contractions due to both these drugs. No corresponding depressor action could be demonstrated when these compounds were tested on the cat's blood pressure

TABLE XI

Relative potencies in stimulating small intestine (Arbitrary scale, C12 = 100)

Compound C2 C4 C5 C6 C7 C8 C9 C10 C11 C12 C18 choline Potency <1 2.0 2.5 3 8 5 12.5 16 40 100 100 25 13 × 10⁴

in intravenous doses up to 10 mg/kg. Table XI gives the estimated relative potencies of the series. A depressor action characteristic of certain histamine-liberators is discussed below.

Search was also made for an atropine-like action, with the response of the cat's blood pressure to acetylcholine as a test object. A dose of 10 mg C10 did not diminish the action of acetylcholine, but rather enhanced it slightly. It appeared possible that the anticholinesterase action of C10 might be masking a feeble atropine-like action. The experiment was therefore repeated in

an eserinized animal, so that inactivation of cholinesterase should be complete before C10 was given, but it was again impossible to demonstrate any depression of the response to acetylcholine by the injection of C10. We obtained a similar result on the isolated guinea-pig's ileum. The contraction of the gut elicited by acetylcholine (2×10^{-9}) , final concentration in the bath) was neither prevented nor diminished by the previous or later addition of C10 to a final concentration of 2×10^{-6} , a concentration sufficient to cause a contraction of the gut due to the C10 itself

Release of histamine

Mention was made at the beginning of this paper that this series is not active in eliciting the delayed depressor response of the cat's blood pressure, characteristic of histamine liberation. This response can, however, be obtained, and Fig. 16

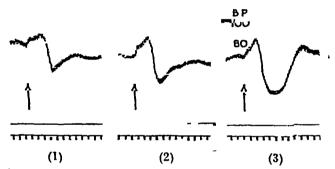


FIG 16—Cat, chloralose Atropine (4 mg), nicotine (7 mg) previously (1) 20 mg C12 i v (2) 1 mg propamidine isethionate i v (3) 5 mg d-tubocurarine chloride i v (12 min between successive tests)

gives a typical illustration of the action of C12, with corresponding effects by d-tubocurarine chloride and propamidine for comparison (cf MacIntosh and Paton, 1949) We have not studied this activity closely since it is so feeble compared with that in paralysing neuromuscular transmission It appears, however, that C12 is a little more active than C10 in this respect

Chronic toxicity

C10—This drug was injected intravenously in a dose of 30 μg /kg into each of three cats, and in a dose of 100 μg /kg into each of three rabbits, six times a week for a period of five to six and a half weeks. During this time there was no consistent change in sensitivity to the drug, although day-to-day variations occurred, sometimes only weakness resulted, at other times a profound paralysis. The veins through which the injections

were made (of the leg in cat, of the ear in rabbit) did not become thrombosed, and the animals maintained their normal weight and health rabbit and one cat, having become deeply curarized after a daily injection of C10, died before their course of six weeks was quite complete, despite artificial respiration. It must be remembered, however, that it is not easy to curarize animals repeatedly without getting occasional casualties, unless elaborate preparations for artificial respiration are made. One cat was killed as soon as it began to sicken at a time that an epidemic of agranulocytic panleucopenia was rife Post-mortem findings showed in this animal only those changes typical of the disease In the other animals there were no evidences of abnormality

C6—C6 was injected into the marginal ear vein of each of three rabbits, in a dose of 10 mg/kg, six days a week for a month. This dose is more than a hundred times the dose which is effective in man. Flushing of the ears was consistently observed, but there was no change in health or weight of the animals, and it was possible in two of the animals to make all the injections into one vein only. Post-mortem findings are given below

If these compounds possessed any chronic toxicity it would probably be of the kind described for another aliphatic dibasic compound "synthalin" (diguanidinodecane) in which parenchymatous changes, particularly of liver and kidney, are prominent. There was no evidence either of these changes or of any other harmful action which could be ascribed to the chronic administration of these drugs.

Post-mortem findings

Sections were taken from lungs, heart, liver, gall bladder, spleen, small and large intestine, kidney, adrenals, testes, lymph glands, bone marrow, brain, and dura mater

In the rabbits, slight peribronchiolar and perivascular lymphocytic infiltration of the lungs was observed, with occasional small areas of ulceration. These changes were also seen in control animals, and were regarded as normal

C10 Cat 1 Chronic abscess of one mesenteric gland, but no intestinal lesion No other abnormality

Cat 2 (Died after daily dose) Pulmonary ocdema No other abnormality

Cat 3 (Sickened during epidemic of panleucopenia) Changes in gut, lymph glands, and marrow, typical of disease No other abnormality

Rabbit 1 Normal.

Rabbit 2 (Died after daily dose) Pulmonary oedema and haemorrhages No other abnormality

Rabbit 3 Not killed Alive and well 1 year later C6 Rabbit 1 Normal

Rabbit 2 Scanty focal patches of infiltration of meninges by lymphocytic cells No other abnormality

Rabbit 3 (Killed 4 months after last injection of C6) The pia arachnoid contains an obvious excess of round cells of lymphocytic type, and similar cells accompany many of the penetrating vessels (cf Rabbit 2) Fairly common areas of perivascular infiltration occur deeply and irregularly within the brain substance. Several foci of neuroglial proliferation are found in the cerebral hemispheres, unrelated to blood vessels, to recent destructive lesions, or to anatomical structures. Their significance is quite obscure. No other abnormality

Antibacterial action

Although there was no reason to suppose that this series of compounds possessed an important antibacterial action, it seemed of interest to determine how far this activity varies with the length of the polymethylene chain. We are much indebted to Dr A T Fuller for testing them for us Table XII gives the concentrations of the various

TABLE XII
Inhibitory concentration of polymethylene bistrimethylammonium salts in mg/100 ml (parts per 100,000)

Compound	Streptococci	Staphylococci	B coli
00 00 00 00 00 00 00 00 00 00 00 00 00	500 500 1,000 500 500 500 750	>500 >1,000 >1,000 >1,000 >1,000 >1,000 >1,000 >1,000	>500 >1,000 >1,000 >1,000 >1,000 >1,000 >1,000 >1,000
C9 C10 C11 C12 C13 C18	500 300, 300 200 150, 100 50 0 3	1,000 1,000 200 100, 200 50 0 8	>1,000 >1,000 >500 100, 200 50 8

compounds which prevent visible growth in 18 hours at 37° C of streptococci, staphylococci, and B coli The antibacterial activity is negligible from C3 to C12, and is only appreciable with C18 Even with the latter compound, however, the activity is only of theoretical interest

The surface tension of aqueous solutions of the bistrimethylammonium series

The change of pharmacological action with polymethylene chain length among these compounds clearly raised the question as to how far this might be due to change in the proportion of hydrocarbon chain to polar groups in the molecule. The measurement of surface tension at an air-water interface provided a suitable method for studying this. Table XIII gives the values obtained

TABLE XIII

Depression of surface tension of water by polymethylene bistrimethylammonium salts (concentration I mg/c.c) Compound C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 C13 C18 $\Delta \gamma \left(\frac{dynes}{cm}\right)$ 04 04 01 13 10 14 06 18 13 13 40 43 19 3

for the depression of the surface tension of water by members of the series, in solutions containing I mg/cc of each salt With most of the compounds there was little depression of the normal surface tension of the water With C12 and C13, however, a distinct depression was observed, which became substantial with C18 Care was taken to control the time for which the surface was allowed to rest undisturbed before making a measurement although the gradual fall of tension with time described for many surface-active substances was only observed with solutions of C18 The depression of tension recorded is therefore to some extent an arbitary one (measured after 10 min had elapsed from first creating the surface), but it appeared to be within 5-10 per cent of the maxi-It follows from these observations that one cannot look to changes in surface activity to explain variations in pharmacological actions of members below about C12

DISCUSSION

Species difference

One of the most remarkable properties of the members of this series, and especially of C10, was the very great variation in their activity according to the species of animal on which they were tested Such a variation is much smaller, and in the reverse sense, with d-tubocurarine chloride Varia. tion of this sort has been described for many other onium salts, but its magnitude for C10, sufficient to make a compound of great activity in the cat appear to be of slight activity in the rat, possesses few parallels The species variation in the sensitivity to histamine is, perhaps, the nearest to it in that cats are relatively sensitive and mice and rats highly resistant But this variation in sensitivity to histamine receives a possible explanation (Emmelin, 1945) in the respective plasma histamine levels of these animals it is reasonable that those with plasmas rich in histamine should be resistant to it, since even cats can be accommodated to sustained histaminaemia No such circulating onium salt has, however, yet been demonstrated, and we

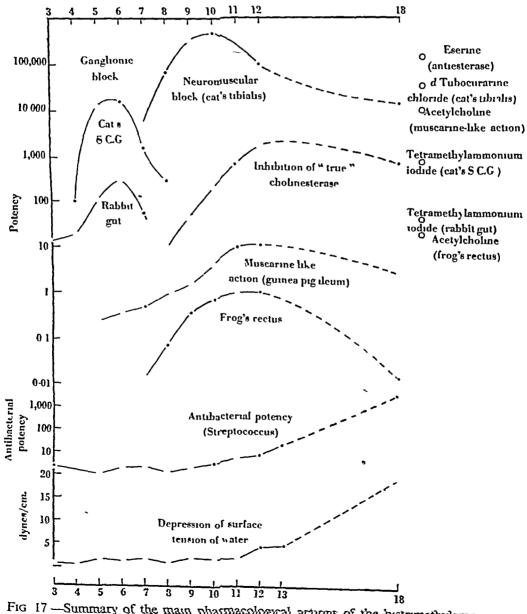
have obtained no evidence of adaptation to the effects of C10

Although, then, there appears to be no ready explanation of the species variation, its practical importance deserves emphasis. Any investigation of new curarizing agents must necessarily be made with more than one species. Further, even with comparative assays of one substance, it may be necessary to establish that the dose-response relations do not vary with different species, and with assays of substances possibly containing several agents, it will be essential to be sure that all the test methods used are sensitive to all the agents involved. The properties of C10 are a strong justi-

fication for including experiments on cats at an early stage in any investigation—particularly since later trials have shown that of all the species tested the cat most closely resembles man (Organe, Paton, and Zaimis, 1949)

The antagonism of C5 and C6 to C10

The characteristics of this antagonism may be briefly summarized as follows it is a reversible antagonism to the action of one active compound displayed by a closely related but inactive homologue. These characteristics, and the other evidence we have presented, are typical of antagonism by competitive inhibition (cf. Woolley, 1947).



of the main pharmacological actions of the bistrimethylammonium series. Abscissa number of carbon atoms in polymethylene chain. Ordinate-logarithmic scale of potency, with arbitrary origins.

For instance, the failure of C5 to antagonize large doses of C10 is readily explained as being due to inability to achieve a sufficiently high ratio of C5 to C10 for successful competition before C5 begins to depress neuromuscular conduction itself

Although this type of antagonism is well known it appears to be a new phenomenon in the field of neuromuscular block. We have not analysed it in any detail. But, for reasons which will be clear from what follows, we do not believe that antagonists of this kind exist for most curarizing compounds, but only for those substances which produce neuromuscular block in the same way as C10

Relation between chemical structure and pharmacological action

We have collated the bulk of the material reported in this investigation in Fig 17. All the main activities of the members of the series are there represented, relating their potency to their chain length. (There is no particular significance in the position of the curve for any one activity on the potency scale, the origin on the scale of ordinates being quite arbitrary, but this scale is logarithmic, so that the slopes of the curves are comparable at all points.) Together with this are plotted the measurements of the depression of the surface tension of aqueous solutions of the compounds

These results may be roughly summarized as showing three maxima for pharmacological activities for ganglionic action at C5-C6, for neuro-muscular blocking action at C10, and a less well defined maximum for stimulation of frog's rectus, muscarine-like, and anticholinesterase activity around C12 These maxima are sufficiently distinct and striking to require separate discussion

The easiest of these to explain is that in the We have, here, bisquaternary region of C12 compounds of increasing chain length whose activities (muscarine-like and in stimulating frog's rectus) steadily approach those of the analogous monoquaternary salts (exemplified by tetramethylammonium ion) Such a tendency is not unexpected, and occurs in other connexions it has been observed with aliphatic diamines, $NH_2(CH_2)_nNH_2$, that the shorter (n=6 or 8) are substrates of diamine oxidase, and the longer members (n=14, 16, or 18) lose this activity but become instead substrates for mono-amine oxidase (Blaschko and Duthie, 1945) Again, MacIntosh and Paton (1949) found that the long-chain diamine with n=16 had a mixed pressor and depressor effect on the cat's blood

pressure, characteristic both of the aliphatic monoamines and of the histamine-liberating diamines On the other hand, as the chain length of these bisquaternary salts increases certain physical properties of the salts (which we have measured by their activity in depressing the surface tension of water) become more prominent. It is interesting that this surface activity begins to appear at C12, the point at which increase in pharmacological activity on frog's rectus and guinea-pig's intestine almost ceases We suggest, therefore, that this maximum near C12 represents the balance-point between an increase in activity as the compounds approximate to an effectively monoquaternary state and a fall in activity as the hydrocarbon moiety of the molecule becomes large enough to influence the action of the compound

The variations of antibacterial potency, reaching a maximum (so far as this series extended) at C18, obviously parallel directly the changes in surface activity. Similar examples, for instance among the alkyl resorcinols, are well recognized, and the relationship between surface activity and antibacterial potency is discussed by Work and Work (1948).

In quite a different category is the maximum at C10, this occurs at a point where changes in surface activity are not yet significant, although the rate of change of potency with chain-length is very rapid round the peak, especially in the C6-7-8 region here alteration of chain length by one carbon atom causes a change in potency by a factor of about 20 times It is of interest that approximately the same distance separates the nitrogen atoms in C10 as in d-tubocurarine chloride and in Bovet's compounds (3381 RP and 3565 R P) despite the differences between C10 and these substances But it may be questioned whether this distance is specific for activity at the neuromuscular junction Thus n=10-11 is optimal for histamine release by diamines (MacIntosh and Paton, 1949), and synthalin (diguanidinodecane) is the most active member of its series in causing hypoglycaemia

The sharpness of the peak raises the question as to why the length of the polymethylene chain should be so critical, and certain comments may be made First, C10 is at least 100 times more active in causing neuromuscular block than tetramethylammonium or trimethyldecylammonium salts (cf Ing and Wright, 1931), it seems necessary to postulate therefore that both ends of C10 are active, presumably (from the symmetry of the molecule) in the same way Further, it is unlikely

TABLE XIV SYNOPSIS OF DIFFERENCES BETWEEN C10 AND d-TUBOCURARINE CHLORIDE

	C10	d-Tubocurarıne chloride
1 Species sensitivity	Cat>Rabbit>Monkey>Mouse>Rat Activity in cat Activity in rat 200	Rat>Mouse>Rabbit>Cat Activity in cat Activity in rat 0 5
2 Muscle selectivity	Paralyses respiratory muscles less than limb muscles in cat	Paralyses respiratory muscles more than limb muscles in cat
3 Character of muscle response to tetanic stimulation during block	Well-sustained contraction	Poorly sustained contraction
4 Effect of KCl or of a tetanus on block	None	Decurarization
5 Effect of antiesterases on block	No antagonism	Decurarization _
6 Effect of C5 on block	Antagonized	No antagonism
7 Effect on striated muscle	Elicits contracture of frog's rectus (and potentiates acetylcholine) Elicits twitch of cat's tibialis	Elicits no contraction of cat's tibialis or of frog's rectus, antagonizes acetylcholine on frog's rectus
8 Effect of d-tubocurarine chloride administered previously	Antagonized (both on cat's tibialis and on frog's rectus)	Potentiated (Also potentiated by C10 previously)
9 Effect of ether anaesthesia	Antagonized	Potentiated
10 Effect on demarcation potential of tibialis muscle	Depressed	Not depressed

that the terminal groups ever approach each other to any great extent, since both carry strong positive charges It is reasonable to suppose, therefore, that the molecule when active is extended on some cell-membrane Finally, it is difficult to avoid the conclusion that the extreme sensitiveness of the activity of the series to chain-length implies some rather specific "fit" between the extended molecule and its effector site tempting then to suppose that the effective distance between the terminal groups (of the order of 10Å, allowing for some freedom of movement of the chain) corresponds to the spacing of some regularly recurring receptor groups, or to the commonest inter-receptor distance in a random array of receptor groups A recurrent acidic residue on a polypeptide chain would provide an array of the type required Such a theory, indeed, explains the shape of the peak at C10, with its rapid cut-off at C6-C7 and slower decline from C12 onwards, for a chain less than the critical length would be entirely unable to straddle the required distance, although a longer chain would, occasionally at least, be able to adopt a configuration with its terminal groups sufficiently close

Similar considerations apply, of course, to the peak for ganglionic activity; but too little is known about such compounds to elaborate the argument Other considerations and anomalies raised by the activities of C5 and C6 will be referred to in another paper. It is worth mentioning here, however, that C10 and C5-C6 provide yet another and remarkable example of the dissociation of activities in neuromuscular and ganglionic block. A dose of C10 more than a hundred times that adequate for neuromuscular block fails to paralyse the superior cervical ganglion, a dose of C5 more than a hundred times that adequate for ganglionic block fails to paralyse the neuromuscular junction

Mode of action of C10

In the following discussion we shall refer only to C10, since of the compounds studied it is the most active as a neuromuscular blocking agent; but we have no reason to doubt, and much evidence to support the presumption, that its neighbours act in the same way. The experiments we have described demonstrate that C10 possesses those properties classically regarded as "curarizing". Like d-tubocurarine chloride, it paralyses neuromuscular transmission only, leaving nervous

conduction unaltered, and permitting the muscle to respond to direct stimulation, it prevents the effect of a close arterial injection of acetylcholine, it does not interfere with the release of acetylcholine. But apart from these resemblances, there are differences which we believe to be sufficiently important to enumerate in some detail (Table XIV)

These differences are more radical than those among, say, d-tubocurarine chloride, its methyl ether or other related alkaloids, and the compounds studied earlier by Bovet and his colleagues Among these there occur variations in potency, in duration of action, in activity in releasing histamine, in ganglionic activity, or in antiesterase But apart from these quantitative differences (which exist also between curare and C10) there appears no evidence that the modes of action of all these compounds differ fundamentally in any way Their actions in causing neuromuscular block, like those of curare, fit conveniently into the theory that they act by competing with the acetylcholine liberated at the nerve terminals for effector sites on the motor end-plate

This is not true for C10 Thus agents delaying the destruction of acetylcholine do not antagonize its action (It might be suggested that this is so because C10 itself already partially inactivates the cholinesterase around the end-plate, but the weakness of C10 as an anticholinesterase, and the absence of any antagonistic action by C10 to that of d-tubocurarine chloride, make it unlikely that more than a small part of the cholinesterase concerned is inhibited by the doses of C10 used) On the other hand, C5 is a successful antagonist, in a fashion strongly suggestive of a competitive inhibition If C10 competes for effector sites with acetylcholine, and if C5 competes with C10 for the same sites, then C5 itself should be able to compete effectively with acetylcholine, and it should hence possess a blocking action at the junction when given in a dose effective in antagonizing C10, this in fact it lacks. Only by additional assumptions about the stability of various drug-effector complexes can the properties of C5 be reconciled with a "competitive" theory of the action of C10 Another important difference lies in the ability of C10 to stimulate skeletal muscle, in concentrations little greater than those in which acetylcholine is effective This, together with the fact that C5 is an antagonist to C10 both when stimulating muscle and when causing neuromuscular block, indicates that the role of C10 is not that of an inactive competitor with acetylcholine but that it is itself active in some respect at the neuromuscular junction

We have described the way in which a muscle, in which partial block has been produced by C10, characteristically sustains its response to tetanization of its motor nerve It is equally characteristic of the partially curarized muscle that it cannot maintain a tetanic response It is out of place to discuss fully this difference here, it may, of course. be due to some peculiar property possessed by d-tubocurarine chloride which has not yet been revealed, but it also raises the possibility that the block produced by C10 is at some other part of the junctional system than that at which d-tubocurarine chloride produces block The strange interaction between the two compounds suggests the same possibility Whatever the explanation of the ability of d-tubocurarine chloride to antagonize C10, and of the inability of C10 to do the same to d-tubocurarine chloride, it is difficult to account for these facts on the supposition that the two compounds block at the same point

The arguments we have presented suggest, therefore,' that C10 produces neuromuscular block by initiating some active response in the end-plate or muscle-fibre, and that this block may arise at a part of the junctional system different from that blocked by d-tubocurarine chloride response of the kind suggested has, indeed, been demonstrated, as a depolarization of the muscle fibre (Brown, Paton, and Vianna Dias, 1949), and this represents an additional distinction of C10 from curare which we have included in Table XIV The relationship of this depolarization to the neuromuscular block remains, however, to be We wish, at the moment, to do no elucidated more than draw attention to the striking differences between the two drugs, and to indicate the possibility of a fundamental divergence in their mode of action

Finally, certain points may be mentioned concerning the differences in chemical structure which underlie the differences between "C10-like" and "d-tubocurarine-like" compounds The information required to make a detailed comparison is not yet available (For references, see Bovet et al, 1949, Paton, 1949) But one may take, as a rough guide, resistance to antiesterases and stimulation of frog's rectus to be typical of C10, and sensitivity to antiesterases and antagonism to acetylcholine on frog's rectus abdominis to be typical of d-tubocurarine chloride It then appears that, of the compounds so far adequately studied which cause neuromuscular block, only tetramethylammonium ion and its trimethyl-alkyl analogues, and succinylcholine (our experience with which agrees with that of Bovet et al, 1949) and its

related aliphatic choline esters, resemble C10, the other compounds, including relatives of d-tubocurarine chloride, the erythroidines, and many of the compounds studied by Bovet and his colleagues, all resemble d-tubocurarine chloride. It is remarkable and suggestive that this division is also between aromatic compounds of widely variable structure on the one hand and, on the other, aliphatic compounds containing methyl-substituted quaternary nitrogen

SUMMARY

- 1 The pharmacological properties of a series of polymethylene α -wbistrimethylammonium iodides with 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 18 carbon atoms in the polymethylene chain have been studied
- 2 The decane derivative (C10) causes neuromuscular block in the cat in doses of 30 µg/kg intravenously, and is the most active member of the series in this respect During such block, the excitability of nerve and muscle is retained, the effects of acetylcholine injected arterially are paralysed, and acetylcholine release by motor nerve terminals is not prevented Tetanization of the motor nerve or injection of acetylcholine or potassium neither diminishes nor deepens the block phase of potentiation of the muscle twitch, fasciculations of the muscle, and repetitive responses to single nerve volleys precedes the block a partial block, tetanization of the muscle leads to a well-sustained contraction
 - 3 In the cat, profound neuromuscular block of the tibialis muscle may be caused by C10, without paralysis of respiratory muscles With d-tubocurarine chloride respiratory paralysis accompanies or may precede paralysis of tibialis
 - 4 C10 is 50-100 times less active by mouth than by intravenous injection. Its activity in cats anaesthetized with chloralose or pentothal is similar to that in the unanaesthetized cat, but ether appears to antagonize it slightly
 - 5 Sensitivity to C10 varies greatly with species in order of decreasing sensitivity, the series is catman rabbit monkey mouse rat
 For d-tubo-curarine chloride by the same tests, the variation in sensitivity is much smaller and decreases ratmouse-rabbit-cat
 - 6 The action of C10 is not antagonized by anticholinesterases. The pentane and hexane homologues (C5, C6) are effective antagonists, the antagonism appears to be by competitive inhibition. Previous administration of d-tubocurarine chloride also reduces the effectiveness of C10

- 7 C10 and its neighbours elicit a contracture of the frog's rectus abdominis muscle, and do not antagonize the contracture elicited by acetylcholine C5 and C6 do not elicit such a contracture, but antagonize the actions both of C10 and of acetylcholine C10 can also elicit a twitch of cat's tibialis muscle if a small dose is given by close arterial injection
- 8 Members of the series, particularly C5 and C6, are able to block ganglionic transmission Higher members possess a weak anticholinesterase action, which is more active against "true" cholinesterase than against "pseudo" cholinesterase They also possess a weak muscarine-like action, a weak power of releasing histamine, and a feeble antibacterial action. No significant atropine-like action, or ability to stimulate autonomic ganglia, was detected. With the exception of C18, members of the series display negligible activity in depressing the surface tension of water.
- 9 The pharmacological properties of the series have been related to the length of the polymethylene chain. This reveals four features (1) a sharp maximum for potency in ganglionic block at C5–C6, (2) a sharp maximum for potency in neuromuscular block at C10, (3) a less well-defined maximum for muscarine-like activity, stimulation of frog's rectus, and anticholinesterase activity about C12, (4) a rise in antibacterial activity and surface activity beginning about C12 and still increasing at C18
- 10 The significance of the species difference, of the competitive antagonism of C5 for C10, of the variation of activity with length of polymethylene, chain, and of the differences between C10 and d-tubocurarine chloride is discussed

It is a pleasure to express our indebtedness to Dr Harold King for his interest and guidance throughout this investigation, and to our colleagues for many fruitful discussions and criticisms

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THE ASSAY OF SUBSTANCES FROM THE ADRENAL MEDULLA

BY

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Much recent work has been devoted to noradrenaline There is evidence that this substance is present in extracts of adrenergic nerves (Euler, 1946, 1948) and liberated when the nerves to the cat's spleen are stimulated (Peart, 1949) The discovery by Holton (1949) that large amounts are present in extracts of adrenal medullary tumours directed attention to the adrenal glands themselves The present investigation was undertaken with the object of applying the methods described by Gaddum, Peart, and Vogt (1949) to blood obtained from the adrenal veins While it has been in progress evidence has been obtained by others that noradrenaline is present in extracts of the adrenal glands (Holtz and Schumann, 1948, Euler and Hamberg, 1949, Goldenberg, Faber, Alston, and Chargaff, 1949, Tullar, 1949), and liberated from these glands when the splanchic nerves are stimulated (Bulbring and Burn, 1949) The results recorded below are in agreement with these conclusions

METHODS

Collection of blood—Cats were anaesthetized with chloralose (40 mg. per kg) after preliminary ether Blood pressure was recorded with a mercury manotheter Most of the cats were eviscerated. The nerves in the mesentery were stimulated after the mesenteric arteries had been tied, but while the portal vein was still open, this transferred blood into the rest of the cat, so that the blood pressure usually remained high in spite of the removal of samples for testing The kidneys were removed or the renal vessels tied The adrenolumbar veins were tied just peripherally to the adrenal glands All other branches of the inferior vena cava below the liver were tied. Both the aorta and the inferior vena cava were tied below the renal vessels, and a cannula inserted in the vena cava just above the ligature When the vena cava was closed by a clip near this cannula adrenal blood flowed to the When this clip was moved to a point above the origin of the adrenolumbar veins, blood flowed out through the cannula and was collected.

Various precautions were taken to prevent clotting and the release of interfering substances. The cannulae were made unwettable with Teddol, obtained from the British Thomson-Houston Co. This was diluted to 5 per cent with CCl4, and the cannulae were rinsed in this fluid and then washed with water Centrifuge tubes were coated with paraffin wax and cooled in ice-water. Heparin (3,000 units per kg) was injected into the cat, and 10 units per ml of blood were placed in each centrifuge tube. Blood was run into these tubes and rapidly centrifuged. Pharmacological tests were applied to the plasma, which was kept in the refrigerator until tested.

Stimulation—The splanchnic nerves were divided early in the experiment. They were stimulated with an alternating current (50 cycles, 5-10 volts)

Preparation of extracts—At the end of some experiments extracts of glands were prepared. After careful dissection the glands were weighed, immersed in 10 ml 0.15N. HCl, ground with sand, and kept in the refrigerator. Shortly before the assay, an aliquot of the supernatant fluid was diluted with saline and the pH was adjusted to the pH was adjusted to the pH was adjusted to the pH was fluid was filtered and the miles used for the test, further dilutions being made if necessary with saline containing ascorbic acid (TV)

Assay—Samples of plasma extracts were compared with solutions of synthetic l-adrenaline (BDH) and dl-noradrenaline hydrochloride (Sterling Winthrop). The results for noradrenaline are expressed in terms of l-noradrenaline, the activity of the d-noradrenaline being neglected

The method used was based on that of Jalon, Bayo and Jalon (1945) Rat's uterus, or colon, is suspended in a solution of the following composition (g/l) NaCl 9 KCl 042 CaCl₂ 006, NaHCO₂ 02, glucose 05 at 30° C Contractions are produced every 2 min by a choline ester, and the assay depends on the inhibition of these contractions by adrenaline or nor-adrenaline

The addition of the choline ester was made automatically by means of the apparatus shown in Fig. 1 it is based on that of Schild (1946, 1947). The uterus or colon is suspended in a small bath (2 ml.). Contractions are produced at intervals of 125 sec. by replacing the solution in the bath by a similar solution

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containing carbachol This substance is more suitable than acetylcholine for this purpose since it is stable even at pH 8 for days, whereas acetylcholine is hydrolysed rapidly in the reservoir. The carbachol solution is allowed to act for 30 sec and is then replaced by the original solution. The concentration of carbachol is 0.5–1.0 μ g per ml for the uterus and 0.05–0.1 μ g per ml for the colon.

The changes are made in the following way A cog wheel, which is rotated by clockwork, makes and breaks a contact every 5 sec At each break a tele-

acts on the muscle for 1 min and is not present in the bath when the carbachol is acting, this causes no serious loss in the sensitivity of the test. In the method used previously the addition of plasma to the bath was apt to cause a variable loss of fluid in froth, so that the addition of a constant amount of choline-ester produced a variable concentration in the bath With the method described here the concentration is constant, and the froth is washed away so that it does not interfere with the contraction

It has been found convenient to conduct tests on the

uterus and colon simultaneously with the arrangement shown in Fig. 1

Uteri from old rats often react slowly and it is sometimes desirable to alter the setting of the apparatus so that the carbachol is in the bath for more than 30 sec Uteri from young rats (4–5 weeks) are generally preferable but may be too thin and weak Satisfactory results have been obtained with such uteri by using both horns tied together, side by side

RESULTS

In a number of experiments blood from the adrenal veins and extracts of glands were tested by the comparatively simple methods used by Gaddum, Peart, and Vogt (1949) It was easy to show in this way that stimulation of the splanchnic nerves, or the injection of acetylcholine,

histamine, or KCl into the arteries, caused the release of large amounts of active substances into the plasma When the plasma was compared with adrenaline by means of the uterus and colon the results were usually discrepant, and the discrepancy could usually be accounted for on the theory that noradrenaline was present as well as Similar evidence was obtained with adrenaline extracts, but none of this evidence was really convincing When the percentage methylation of the mixture is small (ie, when there is a laige excess of noradrenaline) the method works well, as it did in the experiments of Peart (1949) When methylation is more than 50 per cent, the method becomes inaccurate These earlier results were considered

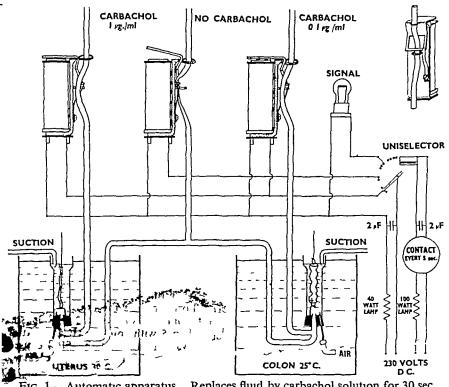


Fig 1—Automatic apparatus Replaces fluid by carbachol solution for 30 sec every 2 min

phone uniselector moves on one place. At suitable intervals this makes contacts which activate telephone relays and these release the compression of rubber tubing and allow solutions to run in at the bottom of the organ bath. A capillary tube permanently connected with a filter pump sucks off excess fluid near the top of the bath, which is wider than the lower part. Each time that the contents of the bath are changed the fluid flows for 5 sec. The height of the reservoir (about 30-40 cm) is adjusted so that the amount of fluid which flows in 5 sec is about 5 times the volume of the bath

When left to itself this apparatus produces a very uniform series of contractions. Solutions of adrenaline or other substances are added to the bath in a volume of 002-02 ml when a signal lights up 1 min before the inflow of carbachol. The adrenaline thus

unsatisfactory and will not be presented in detail

It became clear that better methods were needed. and various methods were tried King (1949) has described a test for adrenaline in which the rat's uterus is bathed in a solution containing more than the usual-amount of potassium We have confirmed the fact that this provides a sensitive test which has the advantage of simplicity On the other hand, it is slower than de Jalon's method. and did not appear to be more sensitive small bath, solutions with a low potassium content inhibit the uterus even when they contain no adrenaline It is not easy to avoid effects due to the potassium content of the solutions tested, and the use of this method was therefore abandoned

The purpose of this paper is to describe experiments by, de Jalon's method using the semi-automatic bath and statistics. The result depended on the measurement of the effects of two doses of the standard preparation $(S_1 \text{ and } S_2)$ and two doses of the unknown preparation $(U_1 \text{ and } U_2)$ and the use of the formulae proposed by Gaddum (1933). The application of this technique to isolated organs was first discussed by Schild (1942), our calculations of the errors were made by the simplified method described by Bliss (1944) and Noel (1945). The results are calculated from differences between effects obtained at about the same time and are thus independent of slow changes in the size of the response

The validity of the calculations depends on the assumption that the effect is linearly related to the logarithm of the dose Various methods of measuring the effect were tried The response to adrenalme depends on the difference between the effect produced under its influence and the effect which would have been produced without it. An estimate of the latter quantity can be obtained by measuring on the drum the last contraction, before the addition of the adrenaline (or other substances) in each of a set of four doses The average of these four figures is taken as the effect of carbachol alone during this time The heights of the contractions in the presence of S and U are calculated as a percentage of this quantity This method of measurement makes allowance for the fact that the response of the uterus to carbachol tends to diminish in the course of an hour or two shows the results of an experiment calculated in this way It also shows the results of a similar experiment with the colon Since the base-line is unstable with this organ the measurements were made from an artificial base-line fixed at an arbitrary level through the bottom of the tracing

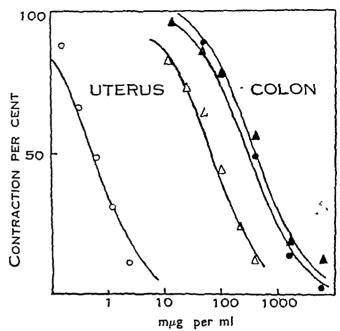


Fig 2—Vertical scale contraction per cent, due to carbachol Horizontal scale concentration of drugs in bath Circles adrenaline Triangles noradrenaline, white rat's uterus, black rat's colon Theoretical curves (see text)

Clark (1937) has drawn attention to the fact that the relation between the dose of acetylcholine or adrenaline and some of their effects on plain muscle can be explained in terms of the mass laws. In the simplest case the curve connecting these quantities is a hyperbola, and the S-shaped curve connecting the effect with log dose has a characteristic slope. The curves drawn in Fig 2-have been calculated on this theory. It will be seen that the observations fit—the curves fairly well except for those showing the effect of adrenaline on the uterus, which would be better fitted by a steeper curve. The calculations might perhaps be based on the assumption that the points lie on

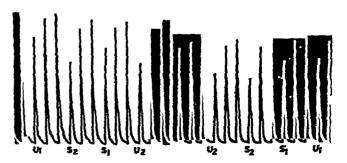


Fig 3—Rat s uterus Exp 50 Carbachol every 2 min Effects of standard adrenaline (1 and 2 mµg) and unknown solution Ratio of doses $S_2/S_1 = U_2/U_1 = 2$

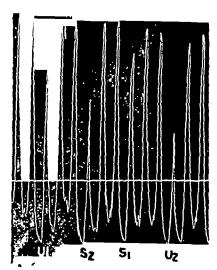


Fig 4 —Rat's colon As Fig 3, but doses of adrenaline 400 and 800 mµg

these curves, but in the present state of knowledge it seems safer to adopt the simpler approximation that the curves are straight lines, this seems to be nearly enough true for effects between 20 and 80 per cent. It is fortunate that the curves for adrenaline and noradrenaline have about the same slope. If this had not been so, the results would have been very difficult to interpret.

TABLE I EFFECTS ON UTERUS (EXP 50)

Sets	Doses		tion before or S	Contraction after U or S		
3613		mm	Mean	mm	Per cent of mean	
1	U ₁ S ₂ S ₁ U ₂	74 73 70 69	71 5	60 46 54 46	84 64 76 64	
2	U ₂ U ₁ S ₂ S ₁	70 65 66 63	66	46 54 44 50	70 82 67 76	
3	U ₂ U ₁ S ₁ S ₂	62 61 61 61	61	41 50 46 37	67 82 75 61	
4	U ₂ S ₂ S ₁ U ₁	62 59 59 60	60	39 37 47 48	65 62 78 80	

Less elaborate methods of measuring the effect can be used in routine tests. The results with the uterus discussed below were actually calculated as percentages, but direct calculations from the heights of the records in mm would probably be equally satisfactory, since the method of calcula-

TABLE II

CALCULATIONS FOR ASSAY ON UTERUS (EXP. 50)

~				(20.1	
<i>N</i> ==	4 Sets of results	1	2	3	4
$S_1 $ $S_2 $	Effects of standard	76 64	76 67	75 61	78 62
U_{\bullet}^{1}	Effects of unknown	84 64	82 70	82 67	- 80 65
D_1	$\begin{array}{c} U_2 - S_2 \\ U_1 - S_1 \end{array}$	0 8	3 6	6	3 2
D_{3}	$U_2 - U_1$	-20	-12	15	15
D_i	$\begin{array}{c} S_2 - S_1 \\ D_1 + D_2 \end{array}$	-12 8	-9 9	-14 13	-16 5
y_2 v_3	$ \begin{pmatrix} D_3 + D_4 \\ D_1 - D_2 \end{pmatrix} $	$\begin{vmatrix} -32 \\ -8 \end{vmatrix}$	-21 -3	-29 -1	31 1
$\overline{T_1}$	$S(y_1)$	Sample dif- ference		35	
T_{z}	S(y2)	Dose differ-		-113	
T_{3}	$S(v_3)$	ence Slope differ-			
I	Log ratio of doses	ence Log 2		—1J 0 3010	
M	T_1I/T_2	Log potency of U/S		-0 09323	
R	100 antilog M	Potency of U (% of S)		.80,68	
b	$T_2/2IN$	Slope		-46 927	
A B	$S(y_1)^2 + S(y_2)^2 + S(y_3)^2$ $S(T_1^2 + T_2^2 + T_3^2)$	_	-	3,68 14,11	5
s	$\sqrt{(A-B/N)/12(N-1)}$	SD of	effect	2 0	566
λ	rsib	S D of log		0 04383	
λ_{M}	$\lambda \sqrt{(1+T_1^2/T_2^2)/N}$	SD of M SD of R		0 02294 4 3	
	(1-antilog λ_M) R or 2 303 λ_M R	SDO	~	• -	
t	$T_3/2s\sqrt{N}$	- }		1 34	
					

slow change in the response of the tissue. In the experiments with the colon the calculations actually were made directly from measurements of the height of the response above an artificial base-line. For this purpose, it is unnecessary that this base-line should be at the bottom of the record and it was placed at any convenient height (cf. Fig. 4). The response of the colon to carbachol was well maintained for six to eight hours, though there was a tendency for the muscle to lengthen gradually. The effect of this change is eliminated by the method of calculation.

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Exp	Uterus			Colon			
	λ	λ <u>w</u>	Coefficient of variation per cent	- λ	$\lambda_{ extbf{M}}$	Coefficient of variation per cent	
40x	0 0778	0 0403	97	0 145	0 0744	18 7	
40y	0 0425	0 0217	52	0 112	0 0572	14 1	
48	0 0615	0 0540	113	0 179	0 0950	24 5	
49	0 0597	0 0314	75	0 101	0 0505	12 3	
51	0 0519	0 0251	59	0 093	0 0470	11 4	
√ ⁵⁰ 52	0 0438	0 0229	54	0 219	0 1125	29 6	
	0 0447	0 0270	64	0 0717	0 0423	10 3	
53(2)	0 0653	0 0382	9 1	0 0529	0 0265	6 3	
53(3)	0 1097	0 0567	14 0	0 164	0 0864	22 0	
Mean	0 06188	0 03527	83 -	0 1264	0 0058	166	

TABLE III
ESTIMATES OF ERROR

The appropriate doses are chosen as the result of preliminary tests. In experiments with the uterus the ratio of the larger doses (S_2 and U_2) to the smaller doses (S_1 and U_1) is 2, with the colon this ratio is 2 or 4. These doses are given in random order, this whole set of results is repeated four times using different random orders of doses. The paper is then varnished, and the contractions measured and the calculations made. Fig. 3 shows the first and last sets of results in experiment 50 with the uterus. Fig. 4 shows one of the four sets of results in experiment 50 with the colon.

Table I shows the measurements made from the tracing shown in Fig 3 and the calculation of the percentage effect, which is shown in the last column

Table II shows the calculation of the result of an assay and its error. In this assay the potency of U was estimated as 80.68 ± 4.3 per cent of that of S. The quantity t is used to calculate whether the slopes of the two curves are significantly different. A standard table of t shows that the value obtained here corresponds to a probability between 0.2 and 0.3, when there are 3(N-1) or 9 degrees of freedom. There is thus no evidence that the lines are not parallel

Table III shows estimates of the error in 9 assays on uterus and colon. The quantity λ gives the best measure of the general accuracy of the test, being independent of the number of sets of results. The mean values were about 0 062 for the uterus and 0 126 for the colon. These figures may

be compared with 0 043 for the assay of histamine on guinea-pig's ileum (Schild, 1942) and 0 033 for the assay of adrenaline on dog's blood pressure (Noel, 1945) The standard error of the actual assays was about 8 3 per cent for the uterus and twice as large for the colon

The test for parallelism of the dose-effect curves was made in all 18 assays P was greater than 005 in every assay except one, and in that one it was only slightly less There was thus no evidence of lack of parallelism

The calculation of concentrations in mixtures

If a solution containing a mixture of adrenaline and noradrenaline is assayed against pure solutions of the two drugs using both uterus and colon, the four results may be used to provide estimates of the concentrations in the mixture, if it is assumed that the effects of the two drugs are simply additive and that no other active substances are present. The logical basis of the formulae used for this purpose is given below. Similar formulae have been devised independently by Euler (1950) and Bulbring (1949)

Let Au, Ac, Nu, and No denote the adrenaline-equivalents and the noradrenaline-equivalents, on the uterus and colon respectively, as determined by assay, and let A and N denote the actual concentrations of the two drugs

Let Ru and Rc denote the ratios of doses of noradrenaline to the equivalent doses of adrenaline, so that Ru = Nu/Au and Rc = Nc/Ac

The adrenaline equivalent of the mixture should be equal to the sum of the adrenaline equivalents of the two drugs present We thus have the two equations

Whence
$$N = RcRu \frac{(Ac - Au)}{(Ru - Rc)}$$

and $A = \frac{AuRu - AcRc}{Ru - Rc}$

Actually Rc is negligibly small compared with Ru, therefore

$$N = (Ac - Au) Rc \tag{1}$$

and
$$A = Au - Ac \frac{Rc}{Ru}$$
 (2)

When the percentage methylation is high it is best to use adrenaline as the main standard preparation, and the equations are given above in a form which is convenient when this is done

The evidence that noradrenaline is present in the mixture depends on the difference between the two estimates of the adrenaline equivalent (Ac and Au) and is only significant when these estimates are significantly different from one another this is so, formula (1) gives a convenient estimate of the concentration of noradrenaline The result with the uterus (Au) can sometimes be taken as a direct estimate of the concentration of adrenaline, equation (2) provides a correction which is negligible when the percentage methylation is high, but becomes important when this percentage falls below 20

When the percentage methylation falls below 10, it is theoretically better to use noradrenaline as the standard preparation and the following equations will then be more convenient They are really the same as those given above

$$A = \frac{(Nu - Nc)}{Ru}$$

$$N = Nc - Nu \frac{Rc}{Ru}$$
(4)

$$N = Nc - Nu \frac{Rc}{Ru} \tag{4}$$

The discussion on equations (1) and (2) also applies mutatis mutandis to equations (3) and (4)

Table IV shows the results by this technique with known mixtures of adrenaline and nor-All these assays were made by the adrenaline statistical procedure outlined above, and their errors have already been discussed It will be seen that the calculations give estimates of the concentrations of adrenaline and noradrenaline in the mixture which are likely to be accurate enough for some purposes

Table V shows the results of the application of this method to extracts of adrenal glands and to blood obtained from the adrenal veins during

TABLE IV ASSAYS OF KNOWN MIXTURES

No of experiment	40x	40y	48	49	51
Adrenaline equiva- lent Uterus Colon	240 1,177	432 1,160	1,438 6,508	2,234 4,860	2,030 3,796
Ratio (Nor Adr) Uterus (Ru) Colon (Rc)	80 1	80 1	500 0 5	37 0 73	-180 1
Adrenaline concentration Calculated Actual Error per cent	225 200 +12 5	417 500 —16 6	1,431 2,000 -28	2,138 2,000 +11	2,009 2,000 +0 4
Noradrenaline con- centration Calculated Actual Error per cent	937 800 +17 1	728 500 +45 6	2,530 2,000 +26 5	1,917 2,000 -41	1,766 2,000 11 7
Total amine Calculated Actual Error per cent	1,162 1,000 +16 2	1,145 1,000 +14 5	3,961 4,000 -1	4,055 4,000 +1 4	3,775 4,000 -5 4
Methylation per cent Calculated Actual	19 20	36 50	36 50	53 50	53 50

stimulation of the splanchnic nerves In all these assays the estimated percentage of methylation was high, and it was necessary to test whether the results could be accounted for on the theory that adrenaline was the only active substance present The evidence against this theory lies in the fact that Ac > Au The fourth line gives estimates of the probability that the observed differences would occur by chance owing to the error of The method by which these figures were calculated may be illustrated by an example In experiment 50 the difference between the logarithms of the two estimates=log 938/556= The standard errors of the logarithms of the two estimates are 0 0229 and 0 1125 The standard error of 0 2201 is thus (Table III) $\sqrt{(0.0229)^2 + (0.1125)^2} = 0.1148$ Therefore t =0.2201/0.1148 = 1.92, and P lies between 0.05 and 01 (9 degrees of freedom) This experiment thus provides no significant evidence of the presence of any active substance in the solution besides adren-The error of the test on the colon was unusually large in this assay On the other hand, experiment 52 provides significant evidence of the presence of some other substance in the extract of

TABLE V
ESTIMATES OF CONCENTRATIONS IN ADRENAL GLANDS AND ADRENAL VENOUS PLASMA

	Adrenal	- 1	Plasma µg per ml		
No of experiment	50	52	53(2)	53(3)	
Adrenaline equiva- lent Uterus Colon Probability of dif- ference	556 938 0 05–0 1	618 877 ca 0 01	2 62 4 78 <0 001	1 66 2 32 0 1–0 2	
Ratio (Nor /Adr) Uterus (Ru) Colon (Rc)	180 0 21	150 0 9	75 0 33	75 0 43	
Calculated concentration Adrenaline Noradrenaline Methylation per cent	555 80 87	613 236 72	2 61 0 71 78 5	1 65 0 28 85 5	

the gland It would clearly not be possible to identify this substance in a mixture by tests of this kind, but, if it is justifiable to assume that it is noradrenaline, then the results give an estimate of the amount present

In experiment 53 a control sample of plasma from the adrenal veins was roughly equivalent on the uterus to 0 005 µg of adrenaline per ml stimulation of the splanchnic nerves, the figure rose immediately to 2 62 µg per ml (sample 2) Stimulation was continued without intermission for 64 min and sample 3 was collected during the last 16 min of this time The results of assays on these two samples are shown in Table V In sample 2 there was significant evidence that adrenaline was not the only active substance present The results with sample 3 were similar, but the evidence was not significant, such results might occur with adrenaline alone The percentage methylation appeared to rise slightly no evidence in this experiment of a loss of the adrenals' powers of methylation even when the nerves were continuously stimulated for over 45 min

DISCUSSION

Straightforward pharmacological methods can be used to identify adrenaline or *nor*adrenaline when either is present alone or in overwhelming excess. When the percentage methylation of a

mixture of these substances is 10 or more, Au gives a direct estimate of A, when it is 10 or less, Nc gives a direct estimate of N. When the percentage is about 10 both these quantities can thus be estimated directly with fair accuracy. In other cases, more complicated methods must be used. If there is doubt about the nature of the active substances present in the mixture the methods described here are not well adapted for their identification. If evidence from other, sources justifies the assumption that these substances are adrenaline and nor adrenaline the formulae give some indication of the percentage methylation.

The presence of *nor*adrenaline in extracts of adrenals is already proved, and the percentage methylation has been estimated by chemical methods. The pharmacological methods have the advantage of sensitivity and may be useful in problems where the concentrations are low. The fact that the methods described here have given similar results to the chemical methods when applied to extracts is evidence in favour of their value when applied to lower concentrations.

The results of experiment 53 show that adrenaline was not the only substance liberated by the Acetylcholine is known to be liberated, but would be hydrolysed in the conditions of these experiments and would not produce the observed effects It would either cause a contraction of the colon within a few seconds of being added to the bath or have no effect at all Histamine is unlikely to be the cause of the discrepancy, since it is comparatively inactive in these tests. The release of noradrenaline is the most likely explanation of this result, which thus confirms the conclusions of Bulbring and Burn (1949) It is, however, clearly, desirable to separate the two substances before tests are made, a method of doing this is in course of development

SUMMARY

- 1 The concentrations of both adrenaline and *nor*adrenaline in a mixture of these drugs can be roughly determined by parallel quantitative assays on the rat's uterus and colon
- 2 The results may be misleading unless they are analysed statistically
- 3 Extracts of cats' adrenals and plasma collected from the cat's adrenal veins during stimulation of the splanchnic nerves both contain some other substance besides adrenaline. The results confirm the view that this is *nor*adrenaline and give an estimate of the amount.

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